Optimization of Bioplastics Production from Cheese Whey

Inês da Silva Farinha

Caparica, December 2009

Universidade Nova de Lisboa

Faculdade de Ciências e Tecnologia

Chemistry Department

Optimization of Bioplastics Production from Cheese Whey

Inês da Silva Farinha

Dissertation presented in partial fulfillment of the Requirements for the Degree of Master in Biotechnology

Supervisiors:

Doctor Luísa Seuanes Serafim, Associated Researcher, REQUIMTE

Professor Maria d'Ascensão Carvalho Miranda Reis, Associate Professor with Aggregation, Faculdade de Ciências e Tecnologia, UNL

Caparica, December 2009

Acknowledgements

I would like to specially thank to my supervisors Dr. Luísa Seuanes Serafim and Professor Maria d'Ascensão Miranda Reis, for their guidance during my research on this project. Their encouragement and support since beginning enable me to complete my work successfully.

I would like to thank to Joana Pais, for her help, support and valuable suggestions through this work.

I want also to thank to all people from the Biochemical Engineering and Processes group laboratory, for their friendship and support.

I would like to thank to Maria A. Prieto from CIB/CSIC and Miguel Arevalo Rodriguez from BIOMEDAL, for their contribution in developing the *E. coli* strains used in this work.

And finally, I want to thank to my parents, for all the patience and support they gave to me.

Abstract

Polyhydroxyalkanoates (PHAs) are polyesters produced by a variety of microorganisms. Due to the similarity of chemical and physical properties with the conventional plastics, and full biodegradability, PHAs constitute one of the best alternatives for synthetic polymers replacement. However, the production costs of these biopolymers are very high when compared to synthetic polymers production. One way to reduce the production costs is the utilization of low cost raw materials, such as industrial wastes and by-products as carbon source. An example of raw material is cheese whey, a by-product form cheese industry rich in lactose (4-5%).

In this work, cheese whey was supplied to *Escherichia coli* strains harbouring the PHB synthesis genes from *Cupriavidus necator* for the production of poly(3-hydroxybutyrate) (PHB). During this study, diverse reactor operating strategies were tested: feeding controlled by pH under oxygen limitation, feeding without oxygen limitation and continuous feeding. The best results were achieved in a fed-batch system with feeding controlled by pH and oxygen limitation, where 44.93% of PHB content, 33.76 g/L of PHB concentration, 78.65 g/L of active biomass concentration and a volumetric productivity of 0.57 g_{PHB}/L.h, were obtained.

Resumo

Polihidroxialcanoatos (PHAs) são poliésteres produzidos por uma grande variedade de microrganismos. Os PHAs constituem uma das melhores alternativas para a substituição dos polímeros sintéticos, devido não só às suas propriedades químicas e físicas, mas também devido ao facto de serem produzidos por via biológica e serem totalmente biodegradáveis. No entanto, os custos de produção destes biopolímeros são muito elevados quando comparados com a produção de polímeros sintéticos. Uma das formas encontradas para reduzir os custos de produção foi a utilização de matérias-primas de baixo custo, como resíduos ou sub-produtos industriais. O soro de leite, um sub-produto proveniente da indústria do queijo com elevado teor em lactose (4-5%), é um exemplo de uma matéria-prima de baixo custo que pode ser utilizada na produção de PHAs.

Neste trabalho efectuou-se o estudo da produção de poli(3-hidroxibutirato) (PHB) a partir do soro de leite, utilizando estirpes de *Escherichia coli* recombinante nas quais foram inseridos os genes para a síntese de PHB da bactéria *Cupriavidus necator*. Durante este estudo foram testadas várias estratégias de operação em reactor semi-contínuo: alimentação controlada pelo pH com limitação de oxigénio, alimentação sem limitação de oxigénio e alimentação contínua. Os melhores resultados foram obtidos no sistema semi-contínuo com a alimentação controlada pelo pH e limitação de oxigénio, apesar de se ter verificado a exaustão de lactose e de oxigénio durante a experiência. Nesta experiência foram obtidos 44.93% de conteúdo em PHB, 33.76 g/L de concentração de PHB e 78.65 g/L de concentração de biomassa activa, e uma produtividade de 0.57 g_{PHB}/L/h.

Contents

Index of Figures

Index of Tables

Nomenclature

%PHB – PHB content (% w/w)

[acetate] – Acetate concentration (g/L)

[ammonia] – Ammonia concentration (g/L)

[butyrate] – Butyrate concentration (g/L)

[formate] – Formate concentration (g/L)

[lactate] – Lactate concentration (g/L)

[lactose] – Lactose concentration (g/L)

[PHB] – PHB concentration (g/L)

[propionate] – Propionate concentration (g/L)

[pyruvate] – Pyruvate concentration (g/L)

[succinate] – Succinate concentration (g/L)

[valerate] – Valerate concentration (g/L)

DCW – Dry Cell Weight, cell concentration (g/L)

DOC – Dissolved Oxygen Concentration (pO_2 (%))

Mn – Average molecular mass

Mw/Mn – Polydispersy index

OD_{600nm} – Optical Density at 600 nm

q_{PHB} – Specific production rate (or specific productivity) ($g_{PHB}/g_X.h$)

r_{PHB} – Volumetric production rate (or volumetric productivity) ($g_{PHB}/L.h$)

rpm – rotation per minute

vvm – gas volume per liquid volume per time (minutes)

X – Active biomass or residual cell concentration (g/L)

$Y_{PHB/lactose}$ – Storage yield ($g_{PHB}/g_{lactose}$)

$Y_{X/lactose}$ – Growth yield ($g_X/g_{lactose}$)

Greek letters

μ - Specific growth rate (h^{-1})

μ_{max} - Maximum specific growth rate (h^{-1})

Abbreviations

BOD – Biochemical Oxygen Demand (ppm)

COD – Chemical Oxygen Demand (ppm)

FDA – U.S. Food and Drug Administration

HB – Hydroxybutyrate

HPLC – High Performance Liquid Chromatography

HV – Hydroxyvalerate

LB medium – Luria-Bertani medium

lcl-PHAs – Long-chain-length

mcl-PHAs – Medium-chain-length PHAs

MR medium – chemical defined medium

PHAs – Polyhydroxyalkanoates

PHB – Poly(3-hydroxybutyrate)

scl-mcl PHAs – scl and mcl monomers copolymers

scl-PHAs – Short-chain-length PHAs

1. Motivation and thesis outline

In the last 50 years synthetic plastics become one of the most utilized materials in many applications. Their versatility, outstanding technical properties (such as high chemical resistance and elasticity) and relatively low price were the main causes of their success (Zinn et al., 2001; Reddy et al., 2003). However, in the recent years, there has been increasing the concern over the harmful effects of petrochemical-derived plastic materials in the environment, especially because they are not biologically degraded. Therefore, it is mandatory to find biodegradable alternative materials to petrol based plastics. Many polymers found in nature were proposed and tested for their possible industrial application and biodegradability, for example, cellulose, starch, polylactate and polyhydroxyalkanoates (PHAs) (Zinn et al., 2001). PHAs were considered strong candidates for biodegradable polymer material because these polymers produced by many microorganisms possess material properties similar to various synthetic thermoplastics currently in use. Moreover, they are completely degraded upon disposal, by microorganisms in various environments such as soil, sea and lake water and sewage (Lee, 1996).

The objective of this work is the production of poly(3-hydroxybutyrate) (PHB), by using recombinant strains of *Escherichia coli* harbouring the *Cupriavidus necator* PHAs biosynthesis genes, from lactose present in cheese whey powder. The success of this process allows the economic valorisation of cheese whey, a residue from dairy industry that needs to be treated before disposal due to its high organic matter content. PHB production from a complex waste like cheese whey comprises three main steps: upstream, fermentation and downstream. This work focuses only the upstream and fermentation steps.

Firstly, batch tests in shake flasks were conducted for the selection of the strain with the highest PHA content and productivity from lactose. Then, other batch tests in shake flask and fed-batch bioreactor assays were conducted with the selected strain, to study the effect of carbon concentration and oxygen limitation on PHB content and productivity in order to optimise this process. Finally different carbon source feeding strategies were evaluated.

2. Introduction

2.1 – Cheese Whey

Whey is the major by-product from the manufacture of cheese or casein bovine milk, representing 80 to 90% of the volume of milk transformed (Ahn et al., 2000) and retaining 55% of milk nutrients (Siso, 1996). Cheese whey is rich in fermentable nutrients such as lactose, lipids and soluble proteins (Table 1), and also contains citric acid, non-protein nitrogen compounds (urea and uric acid) and B group vitamins (Siso, 1996).

Table 1 – Composition of cheese whey (Siso, 1996)

Nutrient	Concentration (% w/v)
Lactose	4.5 – 5
Soluble Proteins	0.6 – 0.8
Lipids	0.4 – 0.5
Mineral Salts (such as NaCl, KCl and calcium salts)	8 – 10(1)
Lactic Acid	0.05

(1) Concentration in percentage of dried extract

According to the procedure used for casein precipitation, the cheese whey produced can be acid (pH<5) or sweet (pH 6-7). Acid whey, also known as salty, has higher salt and lower protein contents than sweet whey. As a consequence of its high salinity, salty whey is more difficult to process and has higher disposal costs than sweet whey (Blaschek et al., 2007; Siso, 1996).

In 2000, in the European Union 6,385.000 tons of cheese were produced resulting in approximately 40,420.800 tons of whey. Around 67% of this whey is used for the production of lactose and animal feed, but the remaining 13,452.000 tons that contain 619.250 tons of lactose constitute a serious disposal problem for the dairy industries (Koller et al., 2008). In Portugal, there were produced 28.146 tons of whey, in 2007 (Instituto Nacional de Estatística, 2008).

Because of the high volumes produced and its high lactose content corresponding to a biochemical oxygen demand (BOD) of 30.000-50.000 ppm and a chemical oxygen demand (COD) of 60.000-80.000 ppm, cheese whey represents an important

environmental problem. As an example, a dairy farm processing 100 tons of milk per day produces approximately the same quantity of organic matter in its effluent as would a town with 55.000 residents. A solution to this water pollution problem is urgent due to the increasing volumes of whey produced and the stricter legislative requirements regarding effluent quality. The bioconversion of whey lactose to marketable products such as ethanol, methane or single cell protein reduces more than 75% of the BOD, but in most cases the resulting effluent is not ready for disposal (Siso, 1996).

To overcome this environmental problem and considering the high content of lactose and proteins, cheese whey may be used as a fermentative feedstock for production of valuable products. Lactose may be used as a carbon source of bacterial strains in the production of bioplastics (like PHAs) (Lee, 1997), ethanol and methane that can be used as a source of energy. Other products such as agricultural fertilizers, animal feeding supplements, organic acids, vitamins, pharmaceutical products and supplements for baby milks can also be obtained by treatment of whey (Siso, 1996). Whey proteins have high nutritional potential and high added commercial value (Zydney, 1998). These proteins can be used as simple protein supplements, for the manufacture of transformed food products because of their functional and technological characteristics (Siso, 1996).

2.2 – Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates (PHAs) are polyesters of various hydroxyalkanoates which are synthesized by numerous microorganisms belonging to the Bacteria and Archea domains (Solaiman et al., 2006), as a carbon and energy reserve material. PHAs are synthesized usually when an essential nutrient is limited (such as oxygen, nitrogen or phosphorus) and in presence of excess carbon source (Lee, 1996). These polymers are accumulated as intracellular granules to levels as high as 90% of the cell dry weight. (Reddy et al., 2003). The occurrence of PHAs in bacteria has been known since 1926, when the French scientist Lemoigne reported the formation of PHB inside *Bacillus megaterium* (Lemoigne, 1927).

2.2.1 – Characteristics of PHAs

Many bacteria can synthesize PHAs with different monomeric composition when the suitable carbon sources are provided. The monomer units in PHAs (Figure 1) are all

in D-(-) configuration due to the stereospecificity of biosynthetic enzymes (Lee, 1996). More than 100 different monomers were identified as constituents of PHAs in various bacteria. However, only a few of these polymers have been produced in sufficient amounts (Zinn et al., 2001) to enable the characterization of their physical properties and to develop potential applications.

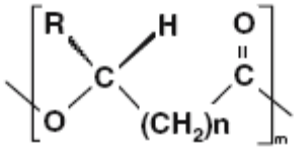

Figure 1 – General structure of PHAs (Solaiman et al., 2006)

PHAs can be mainly divided into three types based on the number of carbon atoms in the monomers. Short-chain-length PHAs (scl-PHAs) include monomer units of C3-C5, medium-chain-length (mcl-PHAs) include monomer units of C6-C14 (Li et al., 2007) and long-chain-length (lcl-PHAs) consist of monomers ranging in size from C14 and more (Luengo et al., 2003).

Due to the diversity of monomeric units, PHAs present a wide variety of physical properties. Scl-PHAs are often crystalline, stiff and brittle, exhibiting thermoplastic-like properties; while mcl-PHAs present lower crystallinity and more elasticity (Reddy et al., 2003), behaving like elastomers or adhesives (Solaiman et al., 2006). The production of PHAs composed of scl and mcl monomers copolymers is quite frequent in nature and scl-mcl PHAs present properties between these two states, depending on the ratio of scl and mcl monomers in the copolymer (Li et al., 2007). Bacterial strains usually produce PHAs with an average molecular mass (Mn) of up to 4.0×10^6 and a polydispersity (Mw/Mn) of around 2.0 (Verlinden et al., 2007). PHAs are also non-toxic, biocompatible and biodegradable thermoplastics. They have a high degree of polymerization and they are optically active, isotactic (stereochemical regularity in repeating units), piezoelectric and insoluble in water (Reddy et al., 2003). These features make them highly competitive with petrochemical-derived plastics.

As mentioned before PHAs can be degraded upon exposure to soil, compost, or marine sediment. Biodegradation is dependent on a variety of factors such as microbial activity of the environment, and the exposed surface area, temperature or pH, polymer composition and crystallinity (Reddy et al., 2003). Biodegradation of PHAs under aerobic conditions results in carbon dioxide and water, whereas in anaerobic conditions

the degradation products are carbon dioxide and methane. Studies have shown that 85% of PHAs were degraded in seven weeks (Reddy et al., 2003).

PHB is a homopolymer of 3-hydroxybutyrate and is the most widespread and best characterized member of PHAs. Like others scl-PHAs, PHB is a partially crystalline polymer with material properties similar to polypropylene. However, industrial applications of PHB have been hampered owing to its low thermal stability and excessive brittleness upon storage (Lee, 1996). The introduction of other 3-hydroxyacid monomers (such as 3-hydroxyvalerate or 4-hydroxybutyrate) in PHB chains, forming copolymers, increases the polymer processability.

2.2.2 – Economical aspects of PHAs production

Renewable resources, like agricultural feedstock or wastes containing sugars and fatty acids as carbon and energy sources can be used for PHAs production. The synthesis and biodegradation of PHAs are totally compatible with carbon cycle (Figure 2). Thus, while for some applications the biodegradability is critical, PHAs receive general attention because their production is based on renewable compounds instead of fossil fuels (Verlinden et al., 2007).

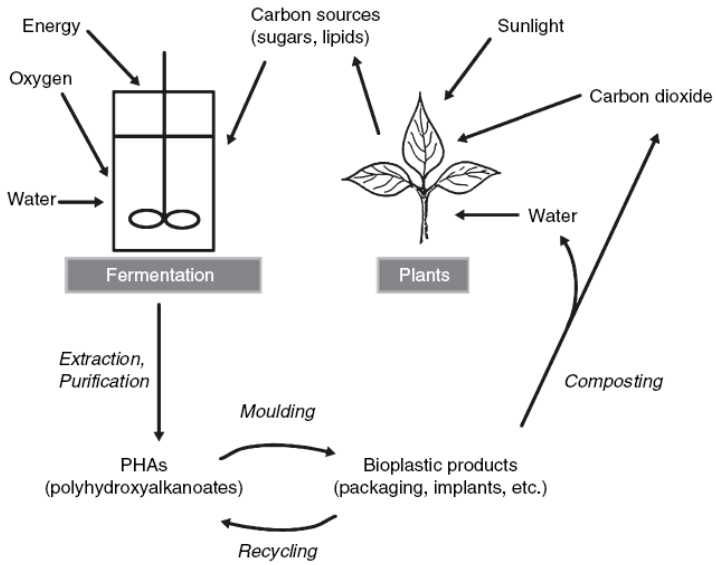

Figure 2 – Life cycle of PHAs (Verlinden et al., 2007)

Life cycle studies of PHAs showed concerns about the production of these biopolymers being more harmful for environment than the production of conventional polymers. According to Harding and co-workers (2007), the major process contributors

to the life cycle impacts are electricity requirements. Nevertheless, considering all the life cycle presented in Figure 2, the production of PHAs is more benefic than petroleum-based polymers. However, the fermentation process to make PHAs is far from optimized, while the production of petrochemical plastics is fully developed (Verlinden et al., 2007).

In spite of the numerous advantages, PHAs are not economically competitive due to its high cost, US$9 per Kg for PHB against US$1 per Kg of synthetic plastics (Biby, 2002). The use of raw materials (such as whey, molasses, malt, soy, vinegar waste and sesame oil) (Solaiman, 2006; Wong et al., 2004), as carbon source for PHAs production can reduce the costs in about 50% (Reis et al., 2003). So, using cheaper carbon sources as basic feedstock could be one of the solutions for decreasing PHAs prices. Despite of the highly developed process of PHA production from wastes, the optimization of the technology for wastes utilization is also urgent. Possible technology for converting wastes in PHAs is the use of recombinant microbial strains to achieve high substrate conversion rates or the selection of open mixed microbial cultures (Reis et al., 2003; Verlinden et al., 2007). With all these improvements, the price of PHAs will be likely becoming US$3-4 per Kg (Choi et al., 1997).

2.2.3 – Applications of PHAs

The majority of possible applications for PHAs are as partially or entirely replacements of petrochemical polymers, especially on packaging and coating applications, such as films, personal hygiene products and adhesives (Reddy et al., 2003; Verlinden et al., 2007). Composites of bioplastics are already used in electronic industry (like mobile phones), agriculture (e.g. for encapsulation of seeds and fertilizers or long term dosage carriers of insecticides and herbicides) and chemical synthesis of optically active compounds (Verlinden et al., 2007).

PHAs have also numerous medical applications, being the main advantage the fact that PHB is biocompatible. R-3-hydroxybutyric acid can be found in blood at concentrations between 0.3 and 1.3 mM and in the cell envelope of eukaryotes (Zinn et al., 2001). For this reason, a biodegradable plastic can be inserted into the human body. In pure form or as composites with other materials, PHAs are used as sutures, orthopedic pins, nerve guides and bone marrow scaffolds; and can be used in future as scaffolds in tissue engineering and as drug carriers (Verlinden et al., 2007). Due to the

strict specifications for compounds used in human body, only some types of PHAs can be used in medical applications; and those used in contact with blood have to be free of bacterial endotoxins (Verlinden et al., 2007). In consequence, extraction and purification methods have to be developed in order to obtain medical PHAs with high purity levels. Recently, the U.S. Food and Drug Administration (FDA) approved the use of PHAs as suture materials in valve heart replacements (Morrow, 2007).

2.3 – Production of Polyhydroxyalkanoates

2.3.1 – Bacterial Strains and the Carbon Sources used

The traditional and commonly used substrates for bacterial production of PHAs are glucose and other saccharides, oils, alcohols and acids (Lee, 1996). Despite being the most commonly used, these substrates contribute largely to the high price of PHAs, since they represent 70 to 80% of the total raw materials costs. Raw materials have a large impact on total operation costs when production scale increase, accounting for as much as 50% of all operation costs (Choi et al., 1997). Currently efforts have been made to substitute these carbon sources by renewable carbon feedstocks like food waste, olive oil mill effluents, frying and palm oil, sugar beet molasses and cheese whey (Verlinden et al., 2007). Some microorganisms can convert methane (Wendlandt et al., 2005), hydrogen (Pohlmann et al., 2007) and carbon dioxide (Jau et al., 2005) to produce PHAs, but with low PHAs contents and low productivities. Unlikely the use of traditional carbon sources, the use of these inexpensive substrates could lead to significant economical advantages (Verlinden et al., 2007).

So far more than 90 genera of archae and eubacteria able to produce PHAs have been detected in aerobic and anaerobic habitats (Zinn et al., 2001). These include *Cupriavidus necator, Alcaligenes latus*, and several strains of methylotrophus, *Pseudomonas oleovorans* and recombinant *Escherichia coli*. All of the aforementioned strains can reach high cell densities with high PHAs content in a short period of time, resulting in high PHAs productivity (Lee, 1996).

Cupriavidus necator (formerly known as *Wautersia eutropha*, *Ralstonia eutropha* or *Alcaligenes eutrophus*) is the most extensively studied PHAs producer organism (Vandamme et al., 2004). In fed-batch cultures, this microorganism can produce PHAs from many different carbon sources such as carbohydrates, ethanol, oleic acid and even

carbon dioxide (Lee, 1996). As mentioned before, PHAs production by a pure culture as *C. necator* from synthetic carbon substrate does not seem the most cost-effective process. Nevertheless, the deep study of PHAs production by this microorganism at enzymatic and genetic level is important in order to optimize the production by recombinant bacteria bearing *C. necator* genes (Verlinden et al., 2007). However *C. necator* showed already promising results in producing PHAs from renewable resources like soybean oil (Kahar et al., 2004).

Escherichia coli is unable of producing PHAs, however it can use several carbon sources, including some substrates that cannot be easily used by most of the microorganisms, such as lactose (Lee et al., 1997). *E. coli* grows fast, even at high temperature, is easy to lyse and is one of the most studied bacteria, being its genome extensively studied. For these reasons, *E. coli* is widely considered as an ideal host for harbouring PHAs producing genes for biopolymer production. Ahn and co-workers (2001) reported a PHB content of 87% of cell dry weight, a PHB concentration of 168 g/L and a productivity of 4.6 $g_{polymer}$/L.h, by a recombinant *E. coli* harbouring the *A. latus* PHAs biosynthesis genes, which is frequently mentioned as the best results ever obtained by a bacteria for PHAs production. The use of recombinant *E. coli* is also advantageous for downstream process, since cells can disrupt easily, contrary to the most natural PHAs producers which are often hard to lyse and contain pathways for PHA degradation (Reddy et al., 2003).

2.3.2 – Bioproduction Process

Currently, there are four methods to produce PHAs: *in vitro*, via PHA-polymerase catalyzed polymerization; and *in vivo* with batch, fed-batch and continuous cultures (Zinn et al., 2001). The fed-batch method is the most used for PHAs production and consists in a two-stage process: an initial growth phase in nutritionally enriched medium to biomass production, followed by a product formation phase in nutrient-depleted medium (Verlinden et al., 2007).

Most of the microorganisms produce PHAs more efficiently when a nutrient is limited (such as nitrogen, phosphorus or oxygen), but not completely depleted, because some of them are required as nutrient for cell survival. A premature limitation of nutrient will result in low final cell and PHA concentrations, resulting in low PHA productivity. If limitation is delayed too long, cells are not able to accumulate much

PHA, resulting in low PHA content and low PHA productivity, even though high cell concentrations can be achieved (Lee, 1996). An example of the effect of a premature limitation is the PHB production by recombinant *E. coli* limited by oxygen, where when the limitation took place too early, cells and PHB concentrations were lower, even with high PHB content (Ahn et al., 2000). Usually, most of the bacterial strains used in these processes are limited by nitrogen, most of the cases without total depletion (Verlinden et al., 2007).

As mentioned before, recombinant *E. coli* strains are able to produce high amounts of biopolymer and to achieve high cell concentrations. In order to support the higher grow rate during growth phase, these strains need large amounts of oxygen and, sometimes, is even necessary an addition of pure oxygen. From the economical point of view, this supplementation of pure oxygen to the system is not economically attractive for PHAs production (Park et al., 2002).

2.3.3 – PHAs production from cheese whey

As mentioned above, cheese whey is rich in lactose and, therefore can be used as carbon source for PHAs production. However only few microorganisms are able to convert directly lactose to PHAs. Microbial conversion of lactose to PHAs can follow three possible strategies (Figure 3). The simplest way is the direct conversion of lactose, but only by a limited number of microorganisms (such as *Hydrogenophaga pseudoflava* and recombinant *E. coli*). Other possible way, but more complex, is the fermentation after enzymatically or chemically hydrolysis step of lactose. The resulting monomers, glucose and galactose, will be used by some microorganisms to produce PHAs (such as *Pseudomonas hydrogenovora* and *Haloferax mediterranei*). Finally, the third possible way involves a first step of anaerobic conversion of lactose to lactic acid and the latter used for PHAs production (by all common PHA producers such as *Alcaligenes latus*) (Koller et al., 2007).

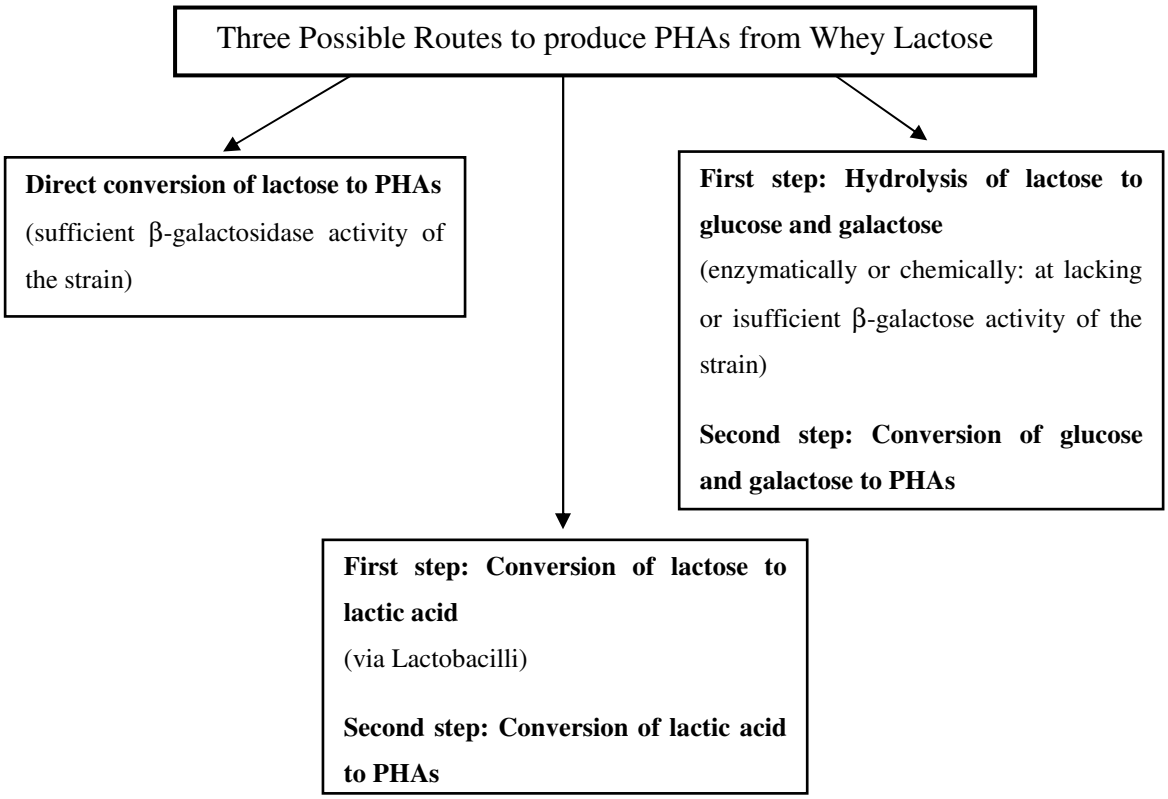

Figure 3 – Possible routes to produce PHAs from whey lactose

Considering these possible ways to produce PHAs from whey lactose, the selection of the process to produce PHA from lactose depends on many factors but mainly on the strains available (Koller et al., 2007).

PHB production metabolism includes the production of acetyl-CoA from sugars and, its subsequent conversion into PHB. PHB production is composed by three enzymatic steps (Verlinden et al., 2007), as shown in Figure 4.

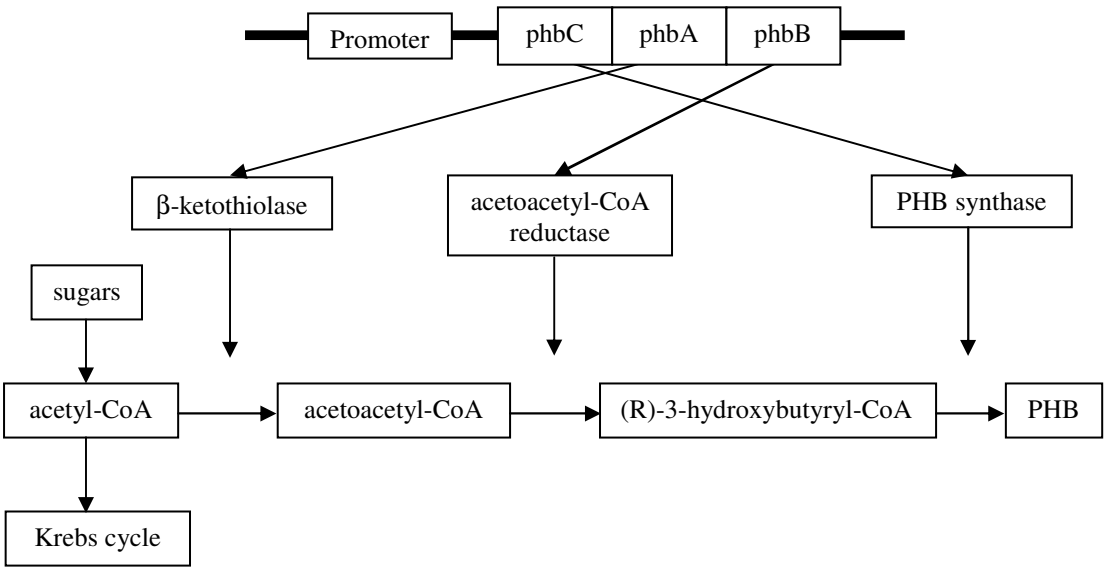

Figure 4 – Biosynthetic pathways to PHB

The first reaction consists on the condensation of two acetyl-CoA molecules into acetoacetyl-CoA by β-ketothiolase (encoded by phbA gene). The second reaction is the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA by an NADPH-dependent acetoacetyl-CoA reductase (encoded by phbB gene). Finally, the (R)-3-hydroxybutyryl-CoA monomers are polymerized into PHB by PHB polymerase (encoded by phbC gene) (Madison et al., 1999; Reddy et al., 2003; Verlinden et al., 2007).

2.3.4 – Downstream Process

After fermentation, bacterial cells with PHAs are normally separated from the medium by centrifugation (Verlinden et al., 2007). Following this separation step, extraction and purification can be done by several methods. There are two possible strategies for extraction of PHAs. One, by using chemicals after the production step, to lyse cells. Other possibility consists on the use of bacterial strains that can lyse easily, by changing external or internal cell parameters, releasing the biopolymer granules. This last strategy is more difficult to implement since not many organisms present a controllable lysis (Li et al., 2007).

Most of the existing methods for PHAs recovery involve the use of organic solvents, such as acetone and chloroform. In organic solvent-based extraction, PHAs are extracted directly from ground biomass by dissolving it in an organic solvent, usually chloroform. After removing cellular components by filtration, the polymer is precipitated in cold ethanol or methanol. PHAs for medical applications, with purity higher than 98% can be achieved with this method. Despite of the higher purity obtained, the use of chlorinated compounds and the necessity of using large volumes of solvents make this procedure economically and environmental unattractive. As an alternative, digestion with sodium hypochloride (Ling et al., 1997) and surfactants (Ramsay et al., 1990), aqueous enzymatic procedures (Holmes et al., 1984), extraction with supercritical CO_2 (Williams et al., 1999) and the employment of less expensive solvents, like sodium hydroxide or potassium hydroxide, can be used as isolating methods, because they are cheaper and efficient with both high PHB yield and high purity (Li et al., 2007).

Recently, the development of systems with self-releasing PHAs capacity began to attract more attention. This system was initially developed for genetically modified *E. coli* strains production and consists in the introduction in bacterial strain the phage lysis

genes, beyond the PHA producing genes (Li et al., 2007). With high producing strains can not be necessary introduce a lysis gene, because high PHA content is beneficial to turns some microbial cells fragile and disrupt easily when accumulating high amounts of PHA (Lee, 1996).

3. Materials and methods

3.1 – *Escherichia coli* Strains

The recombinant *Escherichia coli* strains used in this work were modified by BIOMEDAL (Spain). A genetic construct, plasmid pMAB26, was obtained in order to integrate the PHB producing genes of *Cupriavidus necator* into the chromosome of recombinant *E. coli*, via transposition-mediated mechanism. This new construct contains the PHB operon (with phbC, phbA and phbB genes) inserted in the mini-Tn5 element of plasmid pCNB5 and located under the lactose-inducible Ptrc promoter of this element (Figure 5), allowing a stable integration of these genes and the expression of the corresponding enzymes in the *E. coli* strains. Plasmid pMAB26 was transferred by conjugation to four rifampicin-resistant derivates of *E. coli* strains: BL21(DE3), C, MG1655 and ET8000.

The derivates of the *E. coli* strains obtained were tested for their ability in storing PHAs from lactose. The five best strains were selected for this work: CML 1-1A from BL21 (DE3), CML 2-3A from C, CML 3-1 and CML 3-2A from MG1655 and CML 4-1A from ET8000.

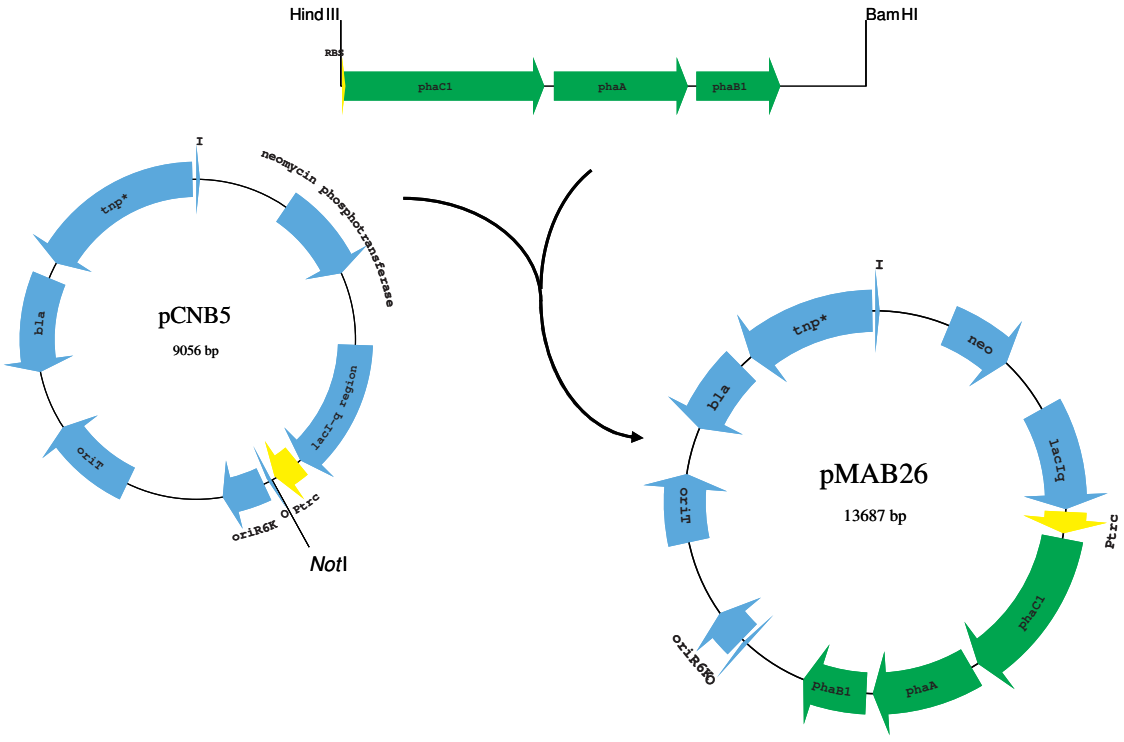

Figure 5 – Construction of plasmids pCNB5 and pMAB26

All recombinant *E. coli* strains were preserved in frozen samples with Luria-Bertani (LB) liquid medium (Table 3) and 20% (v/v) glycerol and conserved at -80ºC.

3.2 – Cheese Whey preparation

3.2.1 – Composition and characteristics

The cheese whey used in this work was supplied by Lactogal (Portugal). The composition and main characteristics of cheese whey determined by the manufacturer are resumed in Table 2.

Table 2 – Characterization of cheese whey powder

Cheese whey	
Fat content (%w/w)	1.21
Protein content (%w/w)	13.62
Lactose content (%w/w)	78.4
Acidity (cm^3 per 100g, NaOH 1M)	11.4
Moisture content (%w/w)	1.8
Specific weight (g/l)	570
Insolubility index (cm^3)	<0.1

3.2.2 – Experimental procedure

Cheese whey preparation was based in Ahn and co-workers (2000) procedure. Cheese whey powder solution was prepared by dissolving between 300 g and 400 g of whey powder in 1 L of distilled water. The whey quantity varied in the several experiments performed in this work, so this information will be detailed in each experiment protocol. In order to remove excessive proteins content, the pH of the solution was adjusted to 4.5 by addiction of 37% (w/v) HCl. The solution was autoclaved at 121ºC for 15 minutes, centrifuged at 8000 g for 15 minutes in sterile bottles to remove aggregates and the pH of solution was adjusted to 6.5 with pellets of NaOH. To remove small protein aggregates, whey solution was filtrated in vacuum and in sterile conditions with filter paper (Whatman no.3). The whey powder solution was kept in sterile conditions in a 1 L Schott previously autoclaved, to avoid contaminations.

3.3 – Experimental set-up

3.3.1 – LB medium experiments

3.3.1.1 – Screening experiments

The screening of the recombinant strain with best PHAs storage capacity was performed with the recombinant strains CML 1-1A, CML 2-3A, CML 3-1, CML 3-2A, CML 4-1A.

3.3.1.1.1 – Medium preparation and composition

The experiments and adaptation steps were carried out in LB liquid medium (Table 3) with 1% (w/v) of lactose. The lactose solution was prepared by dissolving 10.55 g of lactose in 200 mL of distilled water (for 1 L of LB medium). This solution was prepared separately from LB medium to avoid the browning of lactose during sterilization. Then, lactose solution was autoclaved at 121ºC for 15 minutes and after sterilization was joined to LB medium. Also after the LB medium sterilization, 1 mL of Kanamycine (25 g/L) was added to medium.

Table 3 – LB medium (liquid and solid medium) for 1 L of solution

LB medium	
Bactotriptone	10.0 g
Yeast Extract	5.0 g
NaCl	10.0 g
For solid medium:	
Agar	15 g

3.3.1.1.2 – Inocula preparation

For all *E. coli* strains the inocula was obtained after two adaptation steps: the first one in 50 mL flasks with 25 ml of culture broth and the second one in 250 mL flasks with 100 mL of culture broth. One isolated colony from agar plates was used to

inoculate the first adaptation step and 5 mL of the first adaptation step (after 20.5 h of incubation time and an OD_{600nm} of 3.65 with a dilution factor of 1:50) were used for the second one. These adaptation steps cultures were incubated at 37ºC and 200 rpm.

3.3.1.1.3 – Operating conditions

The experiment was performed in 500 ml flasks with 100 ml of culture and without pH control. Cells were incubated at 37ºC and 200 rpm. The medium was inoculated with 2.5 mL of inocula in exponential phase (with 14 h of incubation time).

The assays were run for 75 h and 8 mL samples were taken every two hours until 16 h of incubation. After this period the shake flask was sampled once every day for three days. The samples were analyzed for PHB, biomass and lactose quantification.

3.3.1.2 – Shake flask experiment with the selected strain

E. coli strain CML 3-1, selected as the best PHAs producer, was utilized in this experiment and in the following ones.

3.3.1.2.1 – Medium preparation and composition

The medium used in this experiment and in the inocula was again in LB liquid medium (Table 3) with 2% (w/v) of lactose. The lactose solution was prepared as in section 3.3.1.1.1, but this time, with 21.1 g of lactose dissolved in 200 mL of distilled water.

3.3.1.2.2 – Inocula preparation

The inocula were prepared in two adaptation steps, in 500 mL flasks with 100 ml of culture broth. One isolated colony from agar plates was used to inoculate the first adaptation step and 5 mL of this adaptation step (with 16 h of incubation time) were used as inoculum for the second one. The inocula were incubated at 37ºC and 200 rpm.

3.3.1.2.3 – Operating conditions

The experiment was performed in a 2-L flask with 500 mL of culture and without pH control. Cells were incubated at 37ºC and 200 rpm. The medium was inoculated with 100 mL of inoculum in exponential phase (with 8 h of incubation time).

During this experiment, a pulse of a concentrated solution of lactose (8.5%) in LB medium was supplied to culture every time a pH rise was detected.

The assay was run for 47 h and 15 mL samples were taken every hour. These samples were analyzed for PHB, biomass and lactose quantification.

3.3.2 - Defined medium experiments with lactose

3.3.2.1 – Flask experiment

In this batch experiment, strain CML 3-1 was tested in a defined medium with lactose 2% (w/v).

3.3.2.1.1 – Medium preparation

The experiment and adaptation steps were carried out in defined (MR) medium (Table 4) with 2% (w/v) of lactose. The micronutrients solution was prepared in HCl 1M solution with the composition showed in Table 5. Such as lactose, $MgSO_4.7H_2O$ solution was prepared and sterilized separately from MR medium, in order to avoid the precipitation of phosphates in presence of $MgSO_4.7H_2O$. Proline and Thiamine solution was prepared by dissolving 400 mg of Proline and 6.740 g of Thiamine-HCl, in 20 mL of distilled water and was sterilized by filtration. Proline and Thiamine solution and the Kanamycine solution were also added to MR medium after sterilization.

Table 4 – MR medium with lactose 2% (w/v), for 1 L solution

MR medium with lactose 2% (w/v) (pH 6.7)	
KH_2PO_4	13.5 g
$(NH_4)_2HPO_4$	4.0 g
Citric acid monohydrate	1.8594 g
Micronutrients (Table 5)	10 mL
Lactose Monohydrate	21.1 g
$MgSO_4.7H_2O$	1,4 g
Proline and Thiamine	1 mL
Kanamycine (25 g/L)	1 mL

Table 5 – Micronutrients solution composition

Micronutrients solution in 1 L of HCL 1 N	
$FeSO_4.7H_2O$	10.0 g
$CaCl_2.2H_2O$	2.0 g
$ZnSO_4.7H_2O$	2.2 g
$MnSO_4.H_2O$	0.5 g
$CuSO_4.5H_2O$	1.0 g
$(NH_4)_6MO_7O_{24}.4H_2O$	0.1 g
$Na_2B_4O_7.10H_2O$	0.02 g

3.3.2.1.2 – Inocula preparation

The inocula were prepared as in section 3.3.1.2.2.

3.3.2.1.3 – Operating conditions

The experiment was performed in 250 mL flask with 50 mL of culture and without pH control. Cells were incubated at 37ºC and 200 rpm. The medium was inoculated with 5 mL of inoculum in exponential phase (with 13.5 h of incubation time).

The assay was run for 6 days and 5 mL samples were taken twice or three times a day. These samples were analyzed for PHB, biomass, lactose and ammonia quantification.

3.3.2.2 – Fed-batch experiment

In this experiment, the performance of strain CML 3-1 was tested in a fed-batch reactor with several pulses of lactose.

3.3.2.2.1 – Medium preparation

The fed-batch experiment and flasks adaptation steps were carried out in MR medium with 2% (w/v) of lactose (see section 3.3.2.1.1).

3.3.2.2.2 – Inocula preparation

The inocula were prepared as in section 3.3.2.1.2.

3.3.2.2.3 – Operating conditions

This assay was performed in a 2-L reactor (BioStat® B-Plus, Sartorius, Figure 6) with 1 L of working volume. This experiment was executed at 35ºC, with 100 mL of inoculum (with 13.5h of incubation time) in exponential phase growth. The set-up scheme used in this experiment is shown in Figure 7.

The pH was controlled by automatic addiction of NH_4OH 28% (v/v) solution, when the pH dropped to values lower than 6.5. Dissolved oxygen concentration (DOC, pO_2 (%)) was controlled by automatically increasing the agitation speed from 200 rpm to 700 rpm and by aeration flux (2 vvm of pure air, which contains 21% of oxygen). In this fed-batch experiment, DOC was kept near 40%.

Foam formation was suppressed by addiction of an Antifoam solution, Antifoam A (Fluka). The feeding solution contained 105.5 g of lactose dissolved in 1 L of distilled water. During the day, pulses of 200 mL of feeding solution were added manually, when pH value was above 6.75. During the night, the pulses were automatic with the addition coupled to the pH control, based on Ahn and co-workers (2000) procedure. Every time it rose above 7.0, a lactose pulse was supplied.

Figure 6 - Fed-batch reactor (BioStat® B-Plus, Sartorius)

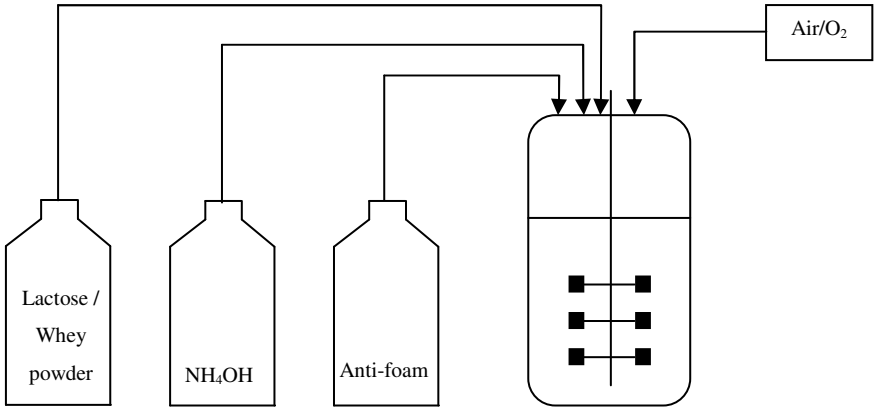

Figure 7 – Fed-batch experiments set-up

The experiment was run for 49 h and 15 mL samples were taken every hour during the day. These samples were analyzed for PHB, biomass, lactose and ammonia quantification.

3.3.3 – Defined medium with cheese whey

3.3.3.1 – Flask experiment

In this batch experiment, the same CML 3-1 strain was tested in a defined medium with cheese whey in a shake flask.

3.3.3.1.1 – Medium composition and preparation

The experiment and adaptation steps were carried out in MR medium with cheese whey. Cheese whey solution was prepared as described in section 3.2.2, by dissolving 300 g of cheese whey in 1 L of distilled water. This solution was added to the MR medium (Table 6) in order to obtain 2% (w/v) of lactose in culture broth.

Table 6 – MR medium with cheese whey, for 1 L solution

MR medium with cheese whey (pH 6.9)	
KH_2PO_4	6.67 g
$(NH_4)_2HPO_4$	4.0 g
Citric acid monohydrate	0.875 g
Micronutrients (Table 5)	5 ml
$MgSO_4.7H_2O$	0.8 g
Cheese Whey solution	

3.3.3.1.2 – Inocula preparation

The inocula were prepared as in section 3.3.1.2.2.

3.3.3.1.3 – Operating conditions

The experiment was performed in 1 L flask with 200 mL of culture and without pH control. Cells were incubated at 37ºC and 200 rpm. The medium was inoculated with 40 mL of inoculum in exponential phase (after 7 h of incubation with an OD_{600nm} of 2.08 with a dilution factor of 1:20). At the end of exponential phase, a pulse of whey solution (17 mL) was added, in order to re-establish the 20 g/L of lactose concentration in culture broth.

This assay was run for 4.5 days. 13 mL samples were taken four times a day, until the end of exponential phase and once a day after the addition of the second whey pulse. Samples were analyzed for PHB, biomass, lactose and ammonia quantification.

3.3.3.2 – Fed-batch experiment with feeding controlled by pH

In this experiment, the performance of strain CML 3-1 was tested in a fed-batch reactor with cheese whey feeding controlled by pH.

3.3.3.2.1 – Medium preparation

The medium used for inocula preparation and in the fed-batch reactor is described in section 3.3.3.1.1. The exception was the feeding solution that contained cheese whey and $MgSO_4.7H_2O$ (6 g/L).

3.3.3.2.2 – Inocula preparation

The inocula were prepared as in section 3.3.3.1.2.

3.3.3.2.3 – Operating conditions

The experimental set-up of this experiment was similar to the used in section 3.3.2.2.3 with two exceptions: pH and DOC profile. pH was controlled to 6.8 and DOC was maintained at 30%. This time, DOC was controlled automatically by changing the stirring speed in the range 200-1000 rpm and by supplying air supplemented with pure oxygen. When OD_{600nm} reached 60, DOC was limited to 10%.

This experiment was run for 62 h and 15 mL samples were taken every hour. Samples were analyzed for PHB, biomass, lactose and ammonia quantification.

3.3.3.3 – Fed-batch experiment with manual feeding

In this experiment, the fed-batch reactor worked with manual feeding.

3.3.3.3.1 – Medium preparation

The medium used for inocula preparation and in the fed-batch reactor is described in section 3.3.3.2.1.

3.3.3.3.2 – Inocula preparation

The inocula were prepared as in section 3.3.3.1.2.

3.3.3.3.3 – Operating conditions

The operating conditions used in this experiment set-up were similar the ones described in section 3.3.3.2.3, but this time without oxygen limitation and pH feed control. DOC was always kept at 60% by stirrer and aeration control. Lactose

concentration was monitorized off-line simultaneously with the experiment. In the beginning of the experiment, 120 mL of cheese whey were added to the reactor. When lactose concentration dropped to values lower than 5 g/L, 90 mL of feeding solution (see chapter 2.3.3.2.1) were supplied manually.

This experiment was run for 23 h. 13 mL samples were taken every two hours during lag phase and every one hour during exponential phase. These samples were analyzed for PHB, biomass, lactose and ammonia quantification.

3.3.3.4 – Fed-batch experiment with manual feeding and oxygen limitation

In this experiment, the performance of strain CML 3-1 was tested in a fed-batch reactor with manually added cheese whey pulses and under oxygen limitation.

3.3.3.4.1 – Medium preparation

The medium used for inocula preparation and in the fed-batch reactor is described in section 3.3.3.2.1. The exception was the cheese whey feeding solution that was prepared by dissolving 400 g of cheese whey in 1 L of MR medium, followed by the procedure described in section 3.2.2.

3.3.3.4.2 – Inocula preparation

The inocula were prepared as in section 3.3.1.2.2 but this time with a double amount of ammonia in MR medium (8.0 g/L of $(NH_4)_2HPO_4$).

3.3.3.4.3 – Operating conditions

The experimental set-up used in this experiment was similar to the described in section 3.3.3.3.3 but now with oxygen limited to 20% after OD_{600nm} reached 100. The inoculum added to medium was incubated after 8 h with an OD_{600nm} of 4.15 with a dilution factor of 1:50.

The experiment was run for 68 h. 15 mL samples were taken every two hours during lag phase. During exponential phase, samples were taken every hour and when a new whey pulse was added. These samples were analyzed for PHB, biomass, lactose, ammonia and organic acids quantification.

3.3.3.5 – Fed-batch experiment with continuous feeding

In this experiment, the performance of strain CML 3-1 was tested in a fed-batch reactor with cheese whey continuous feeding.

3.3.3.5.1 – Medium preparation

The medium used for inocula preparation and in the fed-batch reactor is described in section 3.3.3.4.1.

3.3.3.5.2 – Inocula preparation

The inocula were prepared as in section 3.3.3.4.2.

3.3.3.5.3 – Operating conditions

The experimental set-up used in this experiment was similar to the described in section 3.3.3.3.3 but now with oxygen limited to 30% after OD_{600nm} reached 100 and a continuous feeding strategy was applied. The continuous strategy implemented was the following: in the beginning of the experiment pulse of 30 g/L was added manually, then when lactose concentration reached 24 g/L, the continuous feeding was switched on at a flow rate of 15 g/L/h in exponential phase.

The experiment was run for 131 h and 15 mL samples were taken every two hours. These samples were analyzed for PHB, biomass, lactose, ammonia and organic acids quantification.

3.4 – Analytical Methods

3.4.1 – Cell growth and concentration

Cell growth was monitored online by measuring the optical density of 3 mL of sample, at 600 nm (OD_{600nm}) with a spectrophotometer (Elios α, ThermoSpectronic).

Cell concentration (DCW) defined as the dry weight of cells per litre of culture broth was determined by filtering a sample of culture broth in vacuum with a 0.2 µm membrane previously weight and dried it at 100ºC until obtaining a constant cell dry

weight value, usually after 24h. After drying, the membrane with biomass was weighted again and DCW was determined by the Equation 1:

$$DCW(g/l) = \frac{m_1 - m_2}{V} \quad (1)$$

where m_1 is the mass of the membrane with biomass (g) after drying, m_2 is the mass of the membrane (g) and V is the volume of culture filtrated (L).

3.4.2 – PHB quantification

For quantification of PHB produced, it was used the method proposed by Braunegg and Comeau, with minor modifications introduced by Satoh (Braunegg et al, 1978; Comeau et al., 1998; Satoh et al., 1992). Biomass was collected by centrifuge 1 mL of culture broth at 10000 rpm for 10 minutes. The supernatant was collected for lactose, ammonia and organic acids quantification. The pellet was resuspended in 1 mL of NaCl 0.9% (w/v) and centrifuged again (10000 rpm for 10 minutes). After centrifugation, supernatant was discarded and the pellets were frozen in liquid nitrogen before going to lyophilizer (Telstar, Cryodos). The biomass was lyophilized for 24 h, at approximately -50ºC and 0.07 mbar, in vacuum.

After lyophilization, 2 mg of biomass were resuspended in 1 mL of a solution of methanol with 20% of sulfuric acid and 1 ml of chloroform containing 0.88 mg/ml of heptadecane (internal standard). Then samples were digested in a thermoblock for 3.5 hours and, after cooling, 500 µL of distilled water were added. Samples were shaken for 1 minute in vortex, and then 800 µL of chloroform phase were extracted and transferred to a 2 mL vial with molecular sieves (0.3nm, Merck). Samples were analyzed in gas chromatography (GC, Chrompack SP900), equipped with an ionization flame detector, at 220ºC. 1 µL of chloroform phase was injected in a CPWAX column (60 m, 0.53 mm, 1 µm; Varian), with helium gas as mobile phase, in a split flow manner with a pressure of approximately 100 kPa. The temperatures program for PHB analysis was: 40°C to 100ºC (10°C/min), 100ºC to 175ºC (3ºC/min), 175 to 220ºC (10ºC/min) and 220ºC during 1 minute.

A calibration curve correlating the ratio between the peak areas of HB and heptadecane and the ration between the concentrations was obtained by preparing

standards of HB/HV copolymer (88%/12%; Merck) by subjecting them to the same treatment as the samples. After GC analysis, PHB content and PHB and active biomass (X) concentrations were determined.

3.4.3 – Lactose quantification

For quantification of lactose, samples were centrifuged and the supernatant was diluted 200x with distilled water. After dilution, samples were filtered through a 0.2 µm membrane. For analyzing the lactose present in whey solution, whey samples were diluted 1000x with distilled water.

The concentration of lactose was measured by high-performance liquid chromatography, HPLC (Dionex BioLC) equipped with a Carbopac PA10/MA1 column (4x250) coupled on a amperometric detector. The injection volume was 10 µL and a 100 mM NaOH solution was used as mobile phase, with 0.8 ml/min flux at 30ºC. For lactose quantification, standards containing 0.2 g/L, 0.1 g/L, 0.05 g/L, 0.025 g/L and 0.0125 g/L of lactose were used.

3.4.4 – Organic acids quantification

For quantification of volatile organic acids (lactate, acetate, propionate, butyrate, valerate, formic acid, succinic acid and pyruvate), samples were diluted 20x with distilled water. After dilution, samples were filtered through a 0.2 µm membrane.

The organic acids were determined by high-performance liquid chromatography (HPLC) equipped with an Aminex HPX-87H (Biorad) column coupled to an IR detector. The mobile phase was H_2SO_4 0.01 N, with an elution rate of 0.6 mL/min and an operating temperature of 50 °C.

For organic acids quantification were used standards of 1 g/L, 0.5 g/L, 0.25 g/L and 0.125 g/L.

3.4.5 – Ammonia quantification

The concentration of ammonia was measured using an ammonia electrode (Thermo Electron Corporation, Orion 9512). For ammonia quantification NH_4Cl standards were used.

3.5 – Microscopy analysis

For microscopy visualization of fresh samples, few drops of culture were put in a slide and observed under microscope in phase contrast (Olympus BX51).

For visualization of stain samples, 1 ml of culture broth was centrifuged at 3.000 rpm for 1 minute and supernatant was discarded. It was added 100 µL of nile blue solution to the pellet. This pellet was incubated at 55ºC for 15 minutes. Few drops were put in a slide and were observed in the same microscope using epyfluorescence.

3.6 - Calculations

The PHB content (%PHB) was determined by the Equation 2:

$$\%PHB = \frac{m_{PHB}}{m_{cells}} \times 100\% \quad (2)$$

where m_{PHB} is the amount of PHB (mg) and m_{cells} (mg) is the amount of liophylized biomass in sample.

The PHB concentration ([PHB], g/l) was determined by the Equation 3:

$$[PHB] = \frac{\%PHB}{100} \times DCW \quad (3)$$

The active biomass (X) concentration as follows (Equation 4):

$$X = DCW - [PHB] \quad (4)$$

The PHB volumetric production rate (or volumetric productivity, r_{PHB}) is defined as the maximum amount of PHB produced ($m_{PHB\ max}$) by reactor volume (V) and by time (t). r_{PHB} was determined by the Equation 5:

$$r_{PHB} = \frac{m_{PHB\ max}}{V \cdot t} \quad (5)$$

PHB specific production rate (or specific productivity, q_{PHB}) is defined as the maximum PHB formation ($m_{PHB\ max}$) per maximum active biomass ($m_{X\ max}$) and per time (t). q_{PHB} was determined by the Equation 6:

$$q_{PHB} = \frac{m_{PHB\ max}}{m_{X\ max} \cdot t} \tag{6}$$

The specific growth rate (μ) is defined as the variation of X concentration per time unit and X concentration. The μ maximum value (μ_{max}) was determined from the linear regression slope of the exponential phase of Ln X versus time chart.

The storage yield ($Y_{PHB/lactose}$) and growth yield ($Y_{X/lactose}$) were as follows (Equations 7 and 8):

$$Y_{PHB/lactose} = \frac{m_{PHB}}{m_{lactose\ consumed}} \tag{7}$$

$$Y_{X/lactose} = \frac{m_{X}}{m_{lactose\ consumed}} \tag{8}$$

The lactose consumption rate was determined from the slope between lactose concentration and the growth phase time period.

4. Results and Discussion

4.1 – LB Medium Experiments

4.1.1 – Selection of the strain

The five *E. coli* strains (CML 1-1A, CML 2-3A, CML 3-1, CML3-2A, CML 4-1A) were grown in LB medium with 1% (w/v) of lactose, for the selection of the best PHB producer strain. The performance of the five strains is presented in Table 7 (and in Appendix 8.1.1).

Table 7 – Results obtained in this experiment

Strain	DCW (g/L)	PHB content (%)	[PHB] (g/L)	X (g/L)	$Y_{PHB/lac}$ (g/g)	$Y_{X/lac}$ (g/g)	r_{PHB} (g/L.h)	q_{PHB} (g/g.h)
CML 1-1A	4.73	26.14	1.24	3.49	0.10	0.28	0.016	0.005
CML 2-3A	4.17	40.10	1.67	2.50	0.12	0.17	0.022	0.009
CML 3-1	4.07	52.65	2.14	1.92	0.16	0.14	0.029	0.015
CML 3-2A	3.66	45.15	1.65	2.01	0.14	0.17	0.022	0.011
CML 4-1A	4.27	7.65	0.33	3.94	0.04	0.42	0.004	0.001

The results obtained showed that all the five strains were able to produce PHB from lactose, but CML 3-1 strain was considered the best PHB producer, because presented the highest content (52.65%), polymer concentration (2.14 g/L), volumetric and specific production rates (0.029 g_{PHB}/L.h and 0.015 g_{PHB}/g_X.h, respectively) and storage yield (0.16 $g_{PHB}/g_{lactose}$). CML 3-1 was the only strain with a storage yield higher than the growth yield (0.14 $g_X/g_{lactose}$) meaning that, more lactose was consumed for storage than growth. In opposition, the other four strains drifted more carbon substrate for active biomass formation than for PHB production. The highest growth yield and, consequently, the lowest storage yield were observed for strain CML 4-1A, 0.42 $g_X/g_{lactose}$ and 0.04 $g_{PHB}/g_{lactose}$, respectively. Based on these results the strain selected for the subsequent assays was strain CML 3-1.

4.1.2 – Flask experiments with *E. coli* CML 3-1 strain

In order to determine the maximum PHB storage capacity of strain *E. coli* CML 3-1 from lactose, LB medium was used again but this time with several pulses of 2% (w/v) lactose. When pH rose, which according to Ahn and co-workers (2000) is an indication of lactose depletion, a new pulse of lactose was added to culture, in a total of five pulses. The results of the evolution of lactose, PHB and biomass concentration, as well the storage content and pH are shown in Figure 8.

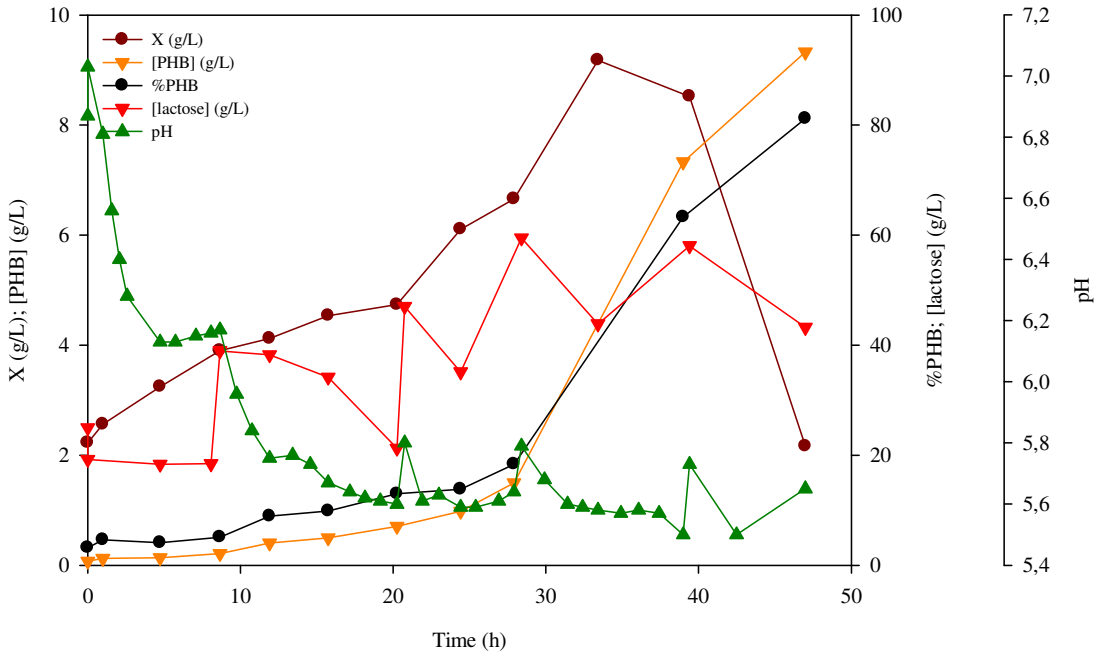

Figure 8 – PHB production from LB medium with lactose by *E. coli* CML 3-1, in shake flask

The values of maximum cell dry weight, PHB storage content, maximum active biomass and polymer concentrations, maximum specific growth rate, storage and growth yields and volumetric and specific productivities performed with *E. coli* CML 3-1 in this work are shown in Table 8. During this experiment, cells grew during the first three pulses but with a minimal PHB quantity production. After the addiction of the fourth pulse (after 28 h of incubation), the active biomass concentration reached its maximum value, 9.18 g/L (at 33.42 h of incubation). After this time a PHB production rate increased sharply and a strong decrease in biomass concentration was observed. At the end of the experiment (after 47 h of incubation), 81.2% of PHB content and 9.32 g/L of PHB concentration were obtained. The storage yield was 0.04 $g_{PHB}/g_{lactose}$ and the growth yield was 0.01 $g_X/g_{lactose}$. Volumetric and specific PHB production rates

Table 8 – Maximum cell dry weight, PHB storage content, maximum active biomass and polymer concentrations, maximum specific growth rate, storage and growth yields and volumetric and specific productivities obtained in the nine experiments performed with *E. coli* CML 3-1 in this work compared with results from the literature (Ahn et al., 2000)

Culture medium	Operational conditions	DCW_{max} (g/L)	PHB content (%)	$[PHB]_{max}$ (g/L)	X_{max} (g/L)	μ_{max} (h^{-1})	$Y_{PHB/lac}$ (g/g)	$Y_{X/lac}$ (g/g)	r_{PHB} (g/L.h)	q_{PHB} (g/g.h)
LB^{k} + lactose 1% (w/v)	Shake flask; Without pH control	4.07	52.65	2.14	1.92	-	0.16	0.14	0.029	0.015
LB^{k} + lactose 2% (w/v)	Shake flask; Without pH control; 5 lactose pulses added	11.58	81.17	9.32	9.18	0.03	0.04	0.01	0.20	0.02
MR^{k} + lactose 2% (w/v)	Shake flask; Without pH control	4.07	19.86	0.81	3.26	-	0.05	0.19	0.006	0.002
MR + 2% lactose 2% (w/v)	Fed-batch; Several pulses and feed controlled by pH; With pH control; DOC > 30%	18.29	25.72	3.46	14.83	0.27	0.01	0.03	0.10	0.007
MR^{k} + cheese whey	Shake flask; Without pH control; 2 whey pulses added	4.17	32.26	1.25	2.92	0.04	0.12	0.27	0.012	0.004
MR + cheese whey	Fed-batch; Feed controlled by pH; With pH control; DOC = 30% (DOC = 10%, when OD=60)	99.37	44.93	33.76	78.65	0.09	0.14	0.50	0.57	0.007
MR + cheese whey	Fed-batch; Manual feed; With pH control; DOC = 60%	48.50	22.22	10.51	37.99	0.15	0.08	0.28	0.46	0.012
MR + cheese whey	Fed-batch; Manual feed; With pH control; DOC = 60% (DOC = 20%, when OD = 100)	138.10	19.76	22.62	114.2	0.40	0.03	0.21	0.33	0.003
MR + cheese whey	Fed-batch; Continuous feed (2 mL per minute in exponential phase and 1 mL per minute in stationay phase); With pH control; DOC = 60%	37.22	63.02	16.21	23.99	0.24	0.13	0.075	0.12	0.015
MR + cheese whey (Ahn and co-workers (2000) results)	Fed-batch; pH feeding strategy; With pH control; DOC = 40% (when DO = 180, CDW = 60 g/L, DOC = 40% » 30% » 15%)	119.50	80	96.20	-	-	-	-	2.57	-

of 0.2 g_{PHB}/L.h and 0.02 g_{PHB}/g_X.h, respectively, and a specific growth rate of 0.03 h^{-1} were obtained (see Figure 24 in Appendix 8.1.2 for calculation). Despite the similar values of PHB storage content obtained in this experiment (81.17%) and obtained by Ahn and co-workers (2000) (80%), the volumetric productivity obtained (0.2 g_{PHB}/L.h) was much lower than 2.57 g_{PHB}/L.h obtained by Ahn and co-workers (2000) (Table 8). This difference on productivity values is probably due to the fact that Ahn and co-workers assay was performed in a fed-batch reactor with defined medium with cheese whey and this experiment was performed in a shake flask with rich medium with lactose. So, the operating conditions related to oxygen availability and medium composition were different and could influence cells performance.

Along the experiment, a gradual lactose accumulation was observed, resulting from the fact that, contrary to the expected, lactose was not depleted when a new pulse of lactose was added. This means that a small increase on pH value can not suggest that lactose was completely consumed from the external medium. Comparing the obtained results with the CML 3-1 screening experiment results (see section 4.1.1), more PHB was produced (9.32 g/L in this experiment and 2.14 g/L in the previous one) since more carbon source was supplied to the microorganisms. In the external medium lactose concentration reached a maximum value of 59.5 g/L.

The morphology of the cells was monitored throughout the experiment by microscope observation under phase contrast (Figure 9). Cells became elongated while PHB granules were accumulated intracellularly. This morphologic modification was also detected by Kim and co-workers (1992) in recombinant *E. coli* strains. PHB granules accumulated at the edge of cells contributing for their elongation.

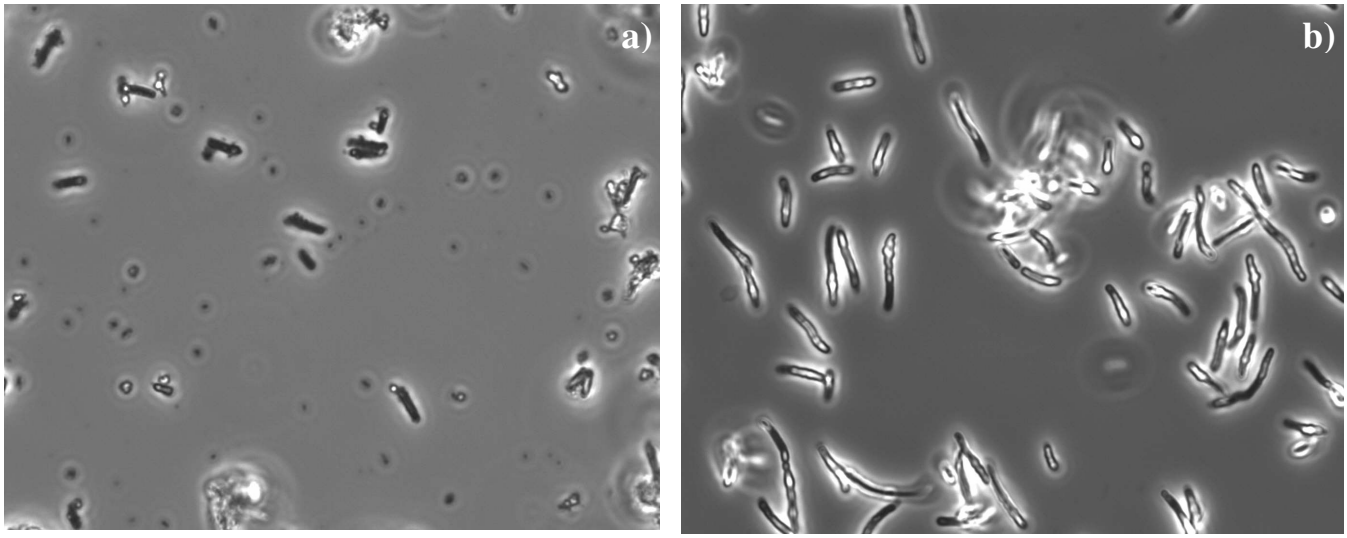

Figure 9 – Fresh samples of *E. coli* CML 3-1 strain lengthening and storing PHB; a) after 7h of incubation, b) after 47h of incubation (1000x)

4.2 – Defined Medium Experiments with Lactose

After testing *E. coli* CML 3-1 in a rich medium, this strain was cultivated in a chemically defined medium, also with 2% (w/v) of lactose as carbon source. The change to a chemically defined medium is related with the raise of industrial production costs, if a rich medium is used, due to its higher complex medium composition.

4.2.1 – Flask experiment

Initially, the strain was tested in MR medium with lactose 2% (w/v) in a shake flask. The results obtained, regarding lactose and ammonia concentration, as well as polymer storage content, OD_{600nm} and pH are shown in Figure 10.

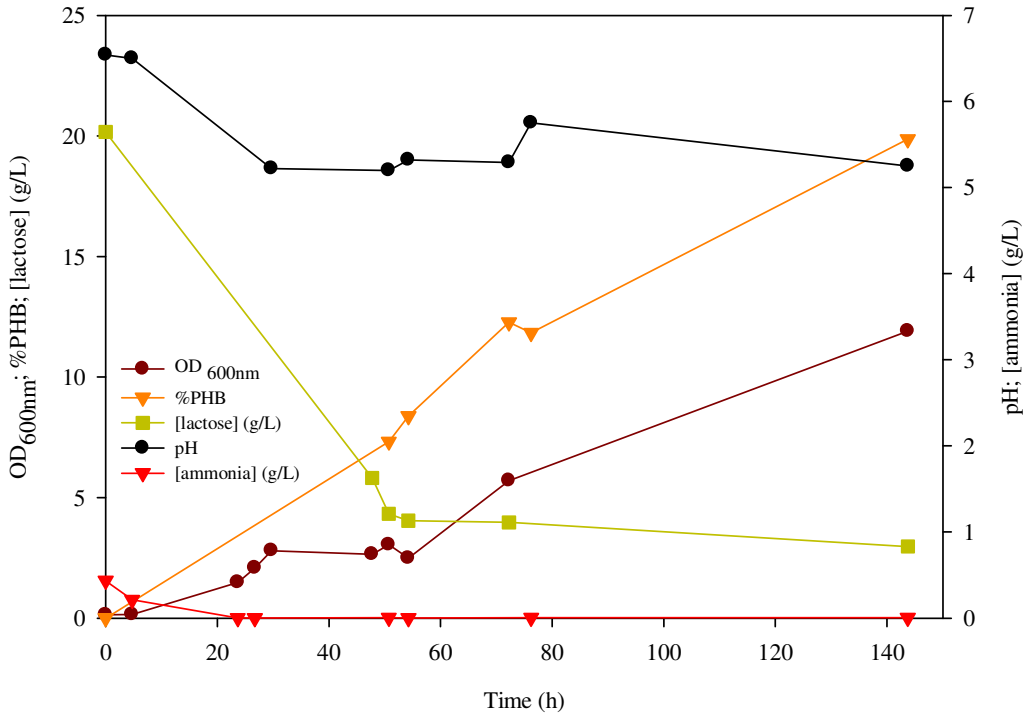

Figure 10 – PHB production from lactose in MR medium by *E. coli* CML 3-1, in shake flask

At the end of the experiment cell concentration reached 4.07 g/L with a PHB content of 19.9% corresponding to 0.81 g/L of PHB and 3.26 g/L of active biomass concentration. Storage and growth yields were 0.05 $g_{PHB}/g_{lactose}$ and 0.19 $g_X/g_{lactose}$, respectively. The volumetric and specific productivities were 0.006 g_{PHB}/L.h and 0.002 g_{PHB}/g_X.h, respectively (Table 8).

These results showed the performance of CML 3-1 strain in MR medium is worse than in LB medium (see section 4.1.1 and Table 8). Thought in this experiment lactose concentration was the double as in the former, the maximum amount of PHB produced (0.81 g/L) was less than a half of the value obtained for LB medium (2.14 g/L).

Despite the low polymer production, the cell dry weight concentration obtained with MR medium was the same as with LB medium (4.07 g/L). This means that in MR medium was achieved a higher active biomass concentration (3.26 g/L) than in LB medium (1.92 g/L). So, in this MR experiment, lactose was consumed preferentially for cell growth. This result is confirmed by comparing growth and storage yields of these two experiments. The growth yield obtained in MR medium (0.19 $g_X/g_{lactose}$) was higher than what was achieved in LB medium (0.14 $g_X/g_{lactose}$) screening experiment, while the

storage yield was the smaller (0.05 $g_{PHB}/g_{lactose}$ for MR medium and 0.16 $g_{PHB}/g_{lactose}$ for LB medium experiment).

In this experiment, the volumetric productivity achieved (0.006 g_{PHB}/L.h) was much lower than 2.57 g_{PHB}/L.h achieved by Ahn and co-workers (2000) (Table 8). Despite this difference, it should be taken in account that this experiment was performed in a shake flask with lactose and Ahn and co-workers (2000) experiment was performed in a fed-batch reactor with cheese whey. This complex substrate is composed by other nutrients (Table 2) that are not present in MR medium with lactose and can enhance bacterial storage and growth.

4.2.2 – Fed-batch experiment

Despite the lower performance of CML 3-1 in shake flask with defined medium and lactose, the PHB production by this strain was tested in a fed-batch system with the same medium. Lactose feeding was controlled by pH. The results obtained regarding lactose, ammonia, PHB and biomass concentration, as well as PHB content, pH, DOC (pO_2) and stirring rate are shown in Figure 11.

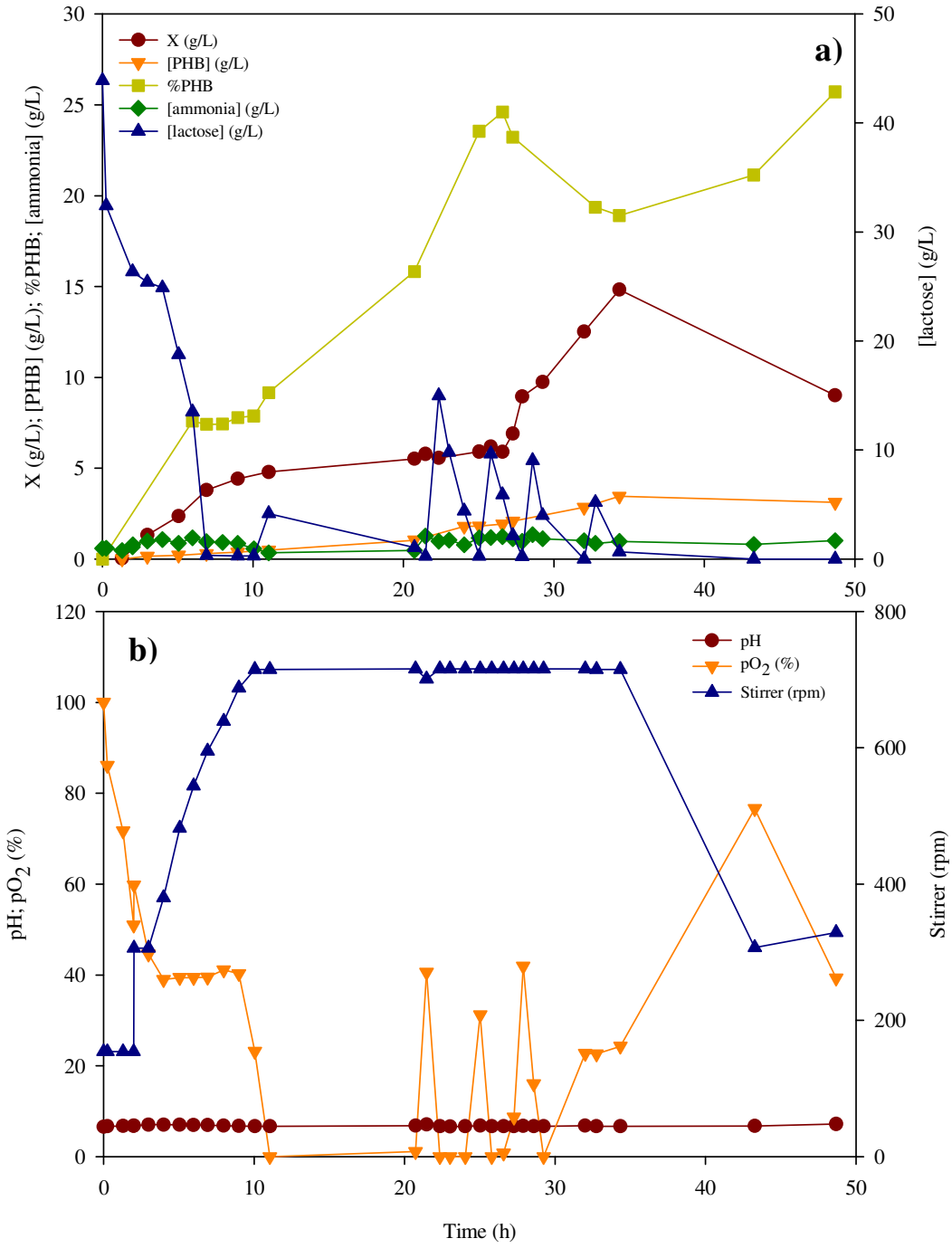

Figure 11 – PHB production in fed-batch reactor with MR medium and lactose, by *E. coli* CML 3-1; a) PHB content and PHB, active biomass, ammonia and lactose concentrations, b) pH, pO_2 and stirrer

Despite the higher amount of lactose supplied and the absence of ammonia limitation in this experiment, the results obtained show a low PHB storage content (25.7 %) and a maximum active biomass of 14.8 g/L (achieved at 34.4 h of operation time).

Storage and growth yields were 0.01 $g_{PHB}/g_{lactose}$ and 0.03 $g_X/g_{lactose}$, respectively. These yields are lower than the yields obtained in the previous experiment (0.05 $g_{PHB}/g_{lactose}$ and 0.19 $g_X/g_{lactose}$, respectively). The PHB volumetric and specific productivities were 0.10 g_{PHB}/L.h and 0.007 g_{PHB}/g_X.h and the maximum specific growth rate was 0.27 h^{-1} (Figure 25, in Appendix 8.2). These productivities are higher than in the previous experiment, since more polymer was produced but still lower than the values obtained by Ahn and co-workers (2000) (2.57 g_{PHB}/L.h) (Table 8).

In this experiment, ammonia was not limiting since it was always above 1 g/L. Due to the higher amount of biomass in the system, the oxygen demand in the fed-batch reactor was higher than in the shake flask and the air supply was not enough for cell growth.

Along operation time, lactose was almost depleted several times. This happened due to pH control, since a lactose pulse was added only when pH rose above its set-point. According to Ahn and co-workers (2000) pH rises only when lactose is absent in medium. In Figure 11, it is possible to observe that lactose was exhausted twice during the operation time and oxygen concentration reached very low values, which both affected cell growth and PHB storage. Between 11h and 21h of operation time, despite the low lactose concentration, cells stopped growing due to oxygen depletion but the PHB storage was enhanced. At the end of the experiment, lactose was again depleted and the active biomass concentration decreased while the PHB storage content increased. Due to the low oxygen concentration, cells did not grow properly.

The optimization of this type of systems must take into account both PHB and cell production. So, it is mandatory to avoid oxygen depletion, in the initial part of the experiment when cells are growing. For this reason in the subsequent fed-batch experiments the air flux supplied was enriched with pure oxygen.

4.3 – Defined Medium Experiments with Cheese Whey

In the previous experiment, low cell and PHB concentrations were obtained in MR medium with lactose. The performance of strain *E. coli* CML 3-1 was tested with MR medium supplement with cheese whey, since the main objective of this work was PHB production from this complex substrate.

4.3.1 – Flask experiment

The storage capacity performance of strain *E. coli* CML 3-1 in MR medium with cheese whey was first studied in a shake flask experiment, as in tests with pure lactose. During this experiment two pulses of cheese whey solution were supplied. The first pulse was added in the beginning of the experiment and the second one in the end of exponential growth phase. The second pulse was added in order to test the strain performance after the end of exponential phase being reached. The results obtained regarding lactose, ammonia, PHB and active biomass concentration, as well as polymer storage content and pH are shown in Figure 12.

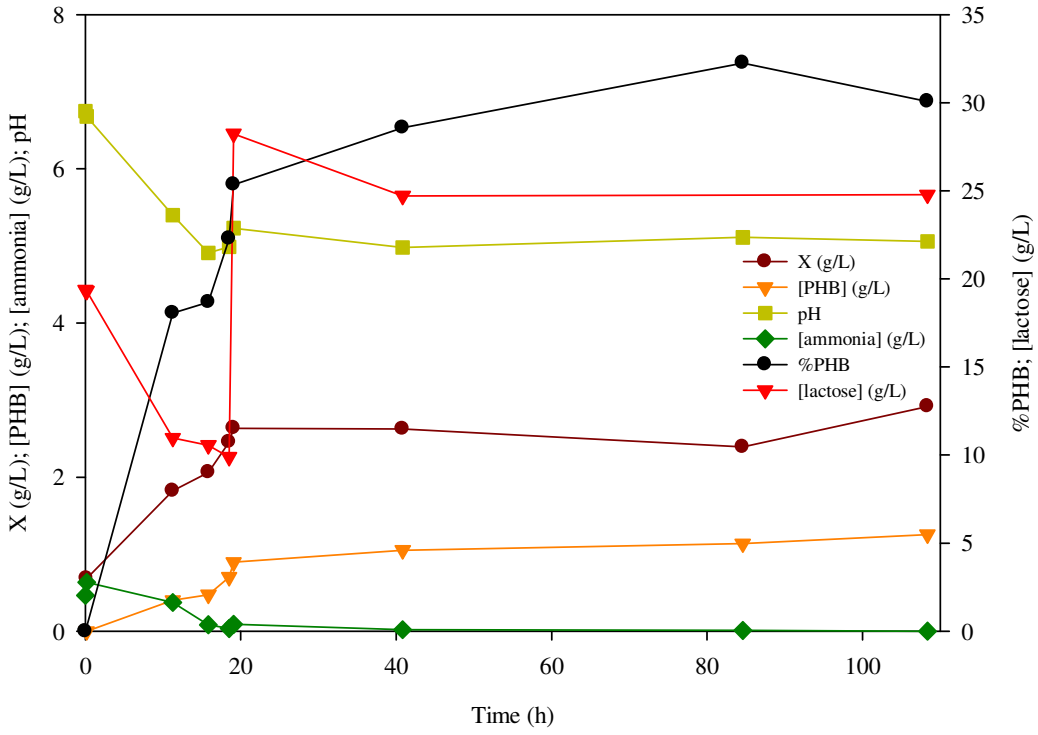

Figure 12 – PHB production by *E. coli* CML 3-1 using whey and MR medium in shake flask

In this experiment, 32.3% of PHB content (achieved at 85h of incubation time), 1.25 g/L of maximum PHB concentration and 2.92 g/L of maximum active biomass concentration (at 108h of incubation time) were obtained. The storage and growth yields were 0.12 $g_{PHB}/g_{lactose}$ and 0.27 $g_X/g_{lactose}$, respectively. The volumetric and specific productivities were 0.012 g_{PHB}/L.h and 0.004 g_{PHB}/g_X.h, respectively. The maximum specific growth rate was 0.04 h^{-1} (Figure 26, in Appendix 8.3.1). The lactose

consumption rates of both pulses were 0.53 g/L/h during the first pulse and 0.16 g/L/h during the second pulse.

Comparing the obtained results with those obtained in MR medium with lactose (see section 4.2.1 and Table 8), it is verified that more PHB was produced (1.25 g/L in this experiment and 0.81 g/L in the experiment with lactose). This higher PHB production also contributed to the higher productivities registered with cheese whey (0.012 g_{PHB}/L.h and 0.004 g_{PHB}/g_X.h). However the values obtained were still lower than those obtained by Ahn et al. (2000) (Table 8).

In opposition to what was observed in the assay with MR medium with lactose (see chapter 4.2.1), the ammonia depletion observed in this experiment (Figure 12) seamed to affect both PHB storage content and cell growth. After the addition of the second whey pulse, almost all lactose consumed was used for polymer storage. In the last 20 h of incubation time, it was observed an increase on active biomass concentration and, consequently a decrease on PHB content. According to Corchero et al. (2001), this probably resulted from the consumption of cells metabolites released to the external medium, because no lactose was consumed since 40 h of incubation.

4.3.2 – Fed-batch experiment with feeding controlled by pH

After the preliminary study in shake flask, a fed-batch experiment in a reactor was accomplished with MR medium and cheese whey, with feeding controlled by pH. In this experiment, the air flux supply was enriched with pure oxygen and, when OD_{600nm} reached the value 60, DOC was limited by 10%. The results obtained regarding lactose, ammonia, PHB and biomass concentration, as well as PHB content, pH, DOC (pO_2), $\%O_2$/air and stirring are shown in Figure 13.

In general, the results obtained in this experiment showed that aeration supplied with pure oxygen contributed for enhancing cells growth, even when an occasionally lactose and oxygen depletions were observed. Unfortunately, due to some operational problems, lactose and oxygen decreased to very low values during the experiment.

A maximum of 33.8 g/L of PHB was obtained at 59 h. The storage of PHB was also enhanced since a PHB content of 44.9% was achieved in this assay, at 56 h of operation time. This is the highest value of PHB content obtained so far in the fed-batch reactor (Table 8). Storage and growth yields were 0.14 $g_{PHB}/g_{lactose}$ and 0.50 $g_X/g_{lactose}$, respectively. The volumetric and specific productivities were 0.57 g_{PHB}/L.h and

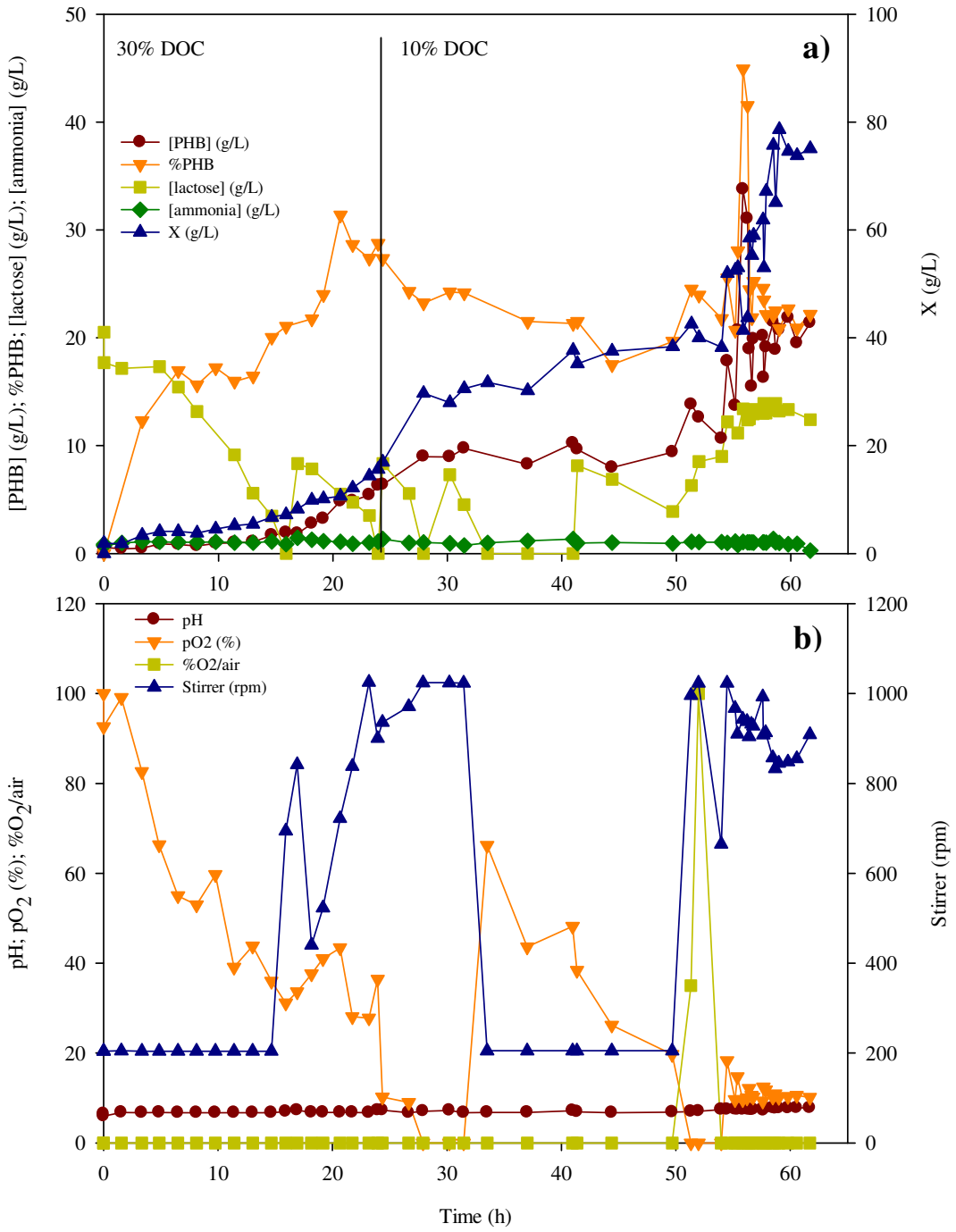

Figure 13 - PHB production from whey in MR medium by *E. coli* CML 3-1, in fed-batch system with feeding controlled by pH; a) PHB content and PHB, active biomass, ammonia and lactose concentrations, b) pH, pO_2, $\%O_2$/air and stirrer

0.007 $g_{PHB}/g_X/h$ respectively and, the maximum specific growth rate was 0.09 h^{-1} (Figure 27, in Appendix 8.3.2). These yields and productivities are higher than in the previous fed-batch experiment with lactose (see section 4.2.2 and Table 8), due to higher amounts of both PHB and biomass produced.

The low values of PHB obtained so far in defined medium supplemented with lactose or cheese whey as carbon substrate could also be a result of the exhaustion of lactose during the experiment. The feeding strategy applied was based on the methodology based on Ahn and co-workers (2000). A new pulse of whey was supplied to the reactor when pH rose above 7.5. But, contrary to what Ahn and co-workers observed, pH took too much time to reach the set point after lactose depletion, remaining the culture without lactose for long time (Figure 14). In the recombinant strain used in this work, the promoter of the PHB operon is induced by lactose (see Section 3.1). This means that synthesis of enzymes needed for PHB production is suppressed if lactose is depleted from the external medium. This situation is observed in both fed-batch experiments with lactose and cheese whey: PHB production stops when no lactose is present in the external medium (Figure 11a and Figure 13a). Taking this fact in consideration, the feeding strategy should be modified in order to always keep a residual amount of lactose in the medium.

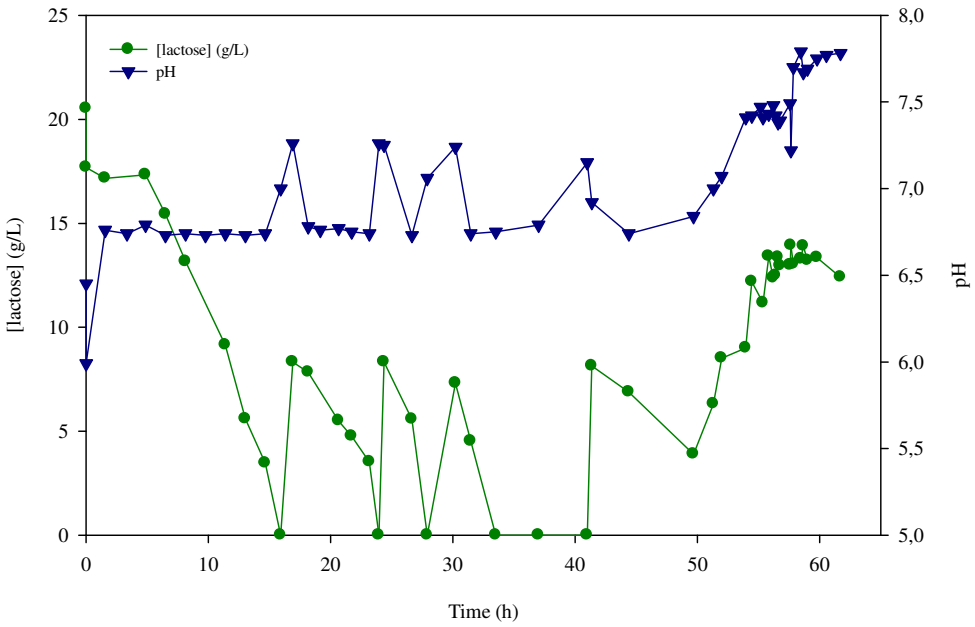

Figure 14 – Feeding controlled by pH strategy

Another possible explanation for weak biomass and PHB production could be the limitation of DOC imposed for promoting PHB production. After 24h of operation time, DOC was limited to 10%, when OD_{600nm} was 68 with a dilution factor of 1:500 (with a cell concentration of 21.94 g/L). This limitation was applied too early, since active biomass did not reach an enough amount for an efficient PHB storage. Ahn and co-workers (2000) tested the value of OD_{600nm} at which the oxygen limitation should be imposed in order to promote PHB production in recombinant *E. coli* and found that an OD_{600nm} of 240 (with a cell concentration of 80 g/L) led to the highest PHB storage content. This OD_{600nm} value was achieved when cell concentration reached 70% of the final cell concentration (Ahn et al., 2000). Moreover, due to equipment limitation in this experiment, the imposition of 10% of DOC was difficult to obtain and most of the time the DOC values decreased to 0%. Due to the oxygen depletion lactose was fermented into organic acids which were released to the culture broth (van Wegen et al., 2001).

Since 50 h of operation time, DOC remains on 10% but lactose begun to accumulate on the culture, with pH always above 7.0 (Figure 13 a) and Figure 14). Possibly this resulted from acids production and their posterior consumption. However, during the lack of oxygen (nearly 30 h of operation time), cells started to ferment lactose into organic acids, such as acetate or lactate. After this 50 h of operation time, cells probably started to consume these acids and pH rose above 7.5 which triggered the fed-batch addition, whey pulses started to be added, causing the accumulation of this carbon source in the medium. During this period cells started to grow and producing PHB very fast, since their concentration almost doubled in 10 h of operation time, the values obtained in the first 50 h.

Despite PHB content (44.93%) and concentration (33.76 g/L) being better than the previous fed-batch experiment with lactose (25.72% and 3.46 g/L, respectively), they are far from the 80% and 96.2 g/L obtained by Ahn and co-workers (2000) (Table 8). Despite the similar feeding strategy implemented in this work the differences observed on cells performance could be related to the operating problems already referred. The differences observed could be also a result from using a different recombinant *E. coli* than Ahn and co-workers (2001), *E. coli* CGSC 4401 containing the *Alcaligenes latus* PHA biosynthesis genes in a plasmid. Strain CML 3-1 contains the genes of a different organism, *C. necator* into the chromosome in order to get a more stable genetic modification.

In order to improve these results, some new experiments were planned regarding the feed strategy. Also additional attention was devoted to the formation of organic acids from lactose during the following assays.

4.3.3 – Fed-batch experiment with manual feeding

Since the strategy of using the whey supply controlled by pH did led to high values of PHB content, a new fed-batch experiment with manual feeding was accomplished. The manual pulses were added when lactose concentration was near 5 g/L. The results obtained regarding lactose, ammonia, PHB and biomass concentration, as well as PHB content, pH, DOC (pO_2), $\%O_2$/air and stirring are shown in Figure 15.

At the end of the experiment, a PHB content of 22.22%, 10.51 g/L and 37.99 g/L of PHB and active biomass concentration, respectively, were obtained. The storage yield was 0.08 $g_{PHB}/g_{lactose}$ and the growth yield was 0.28 $g_X/g_{lactose}$. The volumetric and specific productivities were 0.46 g_{PHB}/L.h and 0.012 g_{PHB}/g_X.h, respectively. The maximum specific growth rate was 0.15 h^{-1} (Figure 28, in Appendix 8.3.3). These yields and productivities are lower than in the previous fed-batch experiment (see section 4.3.2), due to the lower amounts of both PHB and biomass produced.

In this experiment lactose was never exhausted from the culture broth and PHB and cells were continuously produced. As a consequence, the highest value of specific growth rate (0.15 h^{-1}) was so far obtained in a fed-batch reactor (Table 8). The PHB volumetric and specific productivities (0.46 g_{PHB}/L.h and 0.012 g_{PHB}/g_X.h) were lower than in the previous experiment (0.57 g_{PHB}/L.h and 0.007 g_{PHB}/g_X.h). However, it should be underlined that this PHB volumetric storage rate was obtained without oxygen limitation, since the objective of this experiment was mainly to avoid organic acids formation and the repression of PHB synthesis genes. It was expected to obtain a higher amount of PHB if the operation of the reactor was prolonged and an oxygen limitation was imposed. Moreover by increasing the operational time a higher amount of biomass would probably be produced since this assay was stopped when cell were still in the exponential phase. Another aspect to be considered was the long lag phase observed, almost 10 h (Figure 15a). The preparation of the inocula should suffer some modifications in order to avoid a long lag phase.

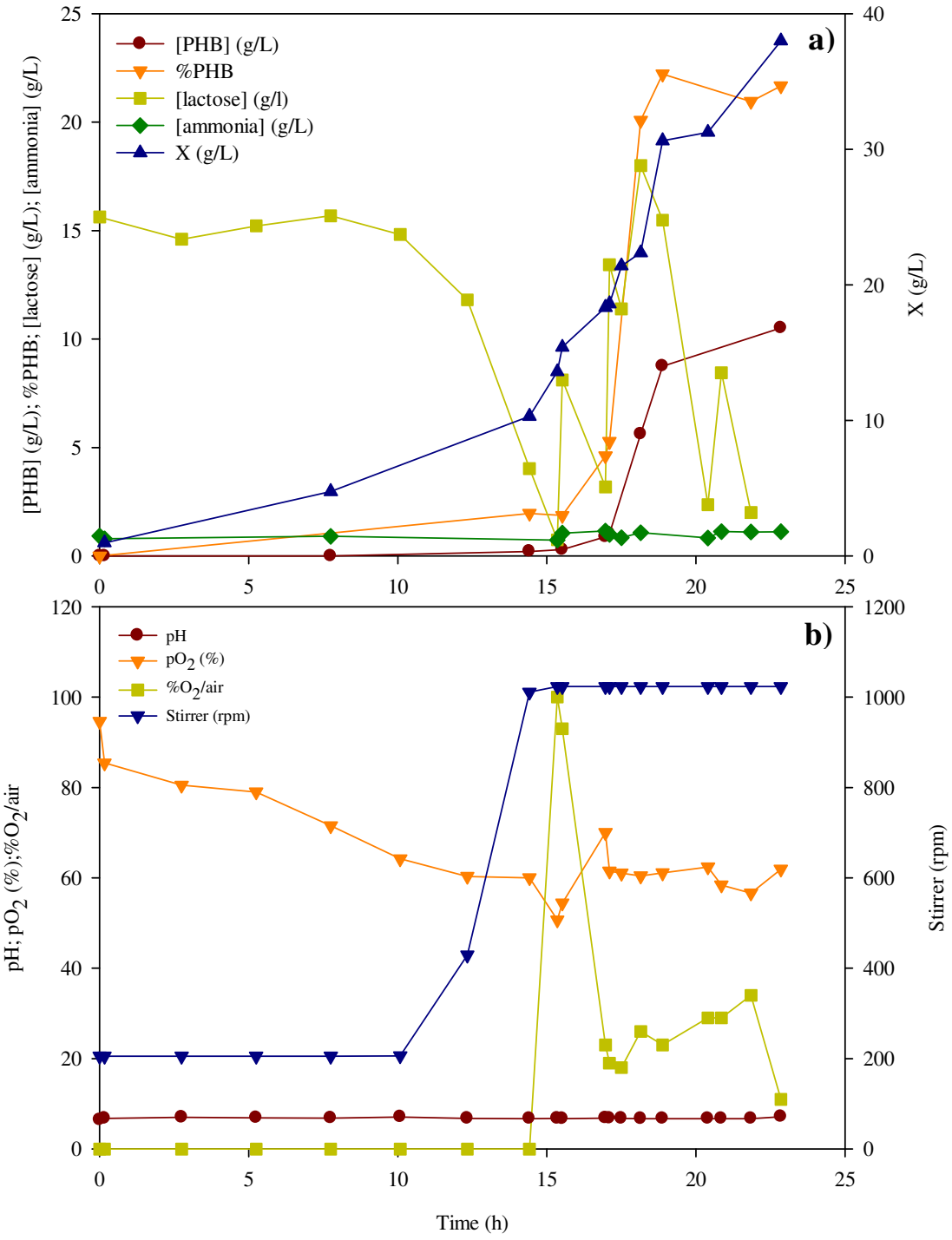

Figure 15 - PHB production from whey in MR medium by *E. coli* CML 3-1, in fed-batch system with manual feeding; a) PHB content and PHB, active biomass, ammonia and lactose concentrations, b) pH, pO_2, $\%O_2$/air and stirrer

4.3.4 – Fed-batch experiment with manual feeding and oxygen limitation

In this experiment the fed-batch reactor worked under the same conditions imposed in the section 4.3.3 but this time, the operational time was prolonged and an oxygen limitation of 20% was imposed when OD_{600nm} reached the value of 100. Cheese whey feeding solution was prepared by dissolving whey in MR medium, in order to obtain a feed solution highly lactose concentrated. The inocula of the reactor were prepared this time with the double of ammonia in order to guarantee that cells were in exponential phase. The results obtained regarding organic acids, lactose, ammonia, PHB and biomass concentration, as well as PHB content, pH, DOC (pO_2), $\%O_2$/air and stirring are shown in Figure 16.

At the end of this experiment 19.76% of PHB content (achieved at 37 h), 22.62 g/L of maximum PHB concentration (achieved at 63 h) and 114.2 g/L of active biomass (achieved at 68 h) were obtained (Table 8). The storage and growth yields were 0.03 $g_{PHB}/g_{lactose}$ and 0.21 $g_X/g_{lactose}$, respectively. The volumetric and specific PHB storage rates were 0.33 g_{PHB}/L.h and 0.003 g_{PHB}/g_X.h and, the maximum specific growth rate was 0.40 h^{-1} (Figure 29, in Appendix 8.3.4). The maximum cell concentration obtained (138 g/L) was higher than the value of final cell concentration, 119.5 g/L, obtained by Ahn and co-workers (2000). These results showed that this strategy is adequate to improve cells growth, but inhibits the polymer storage.

In Figure 16a shows that the lag phase was reduced from 15 h (see section 4.3.3) to only 5 h. This confirmed that the increased of ammonia concentration used in the preparation of the inocula was beneficent for the biomass growth and the cells were really in the exponential phase when the reactor was inoculated.

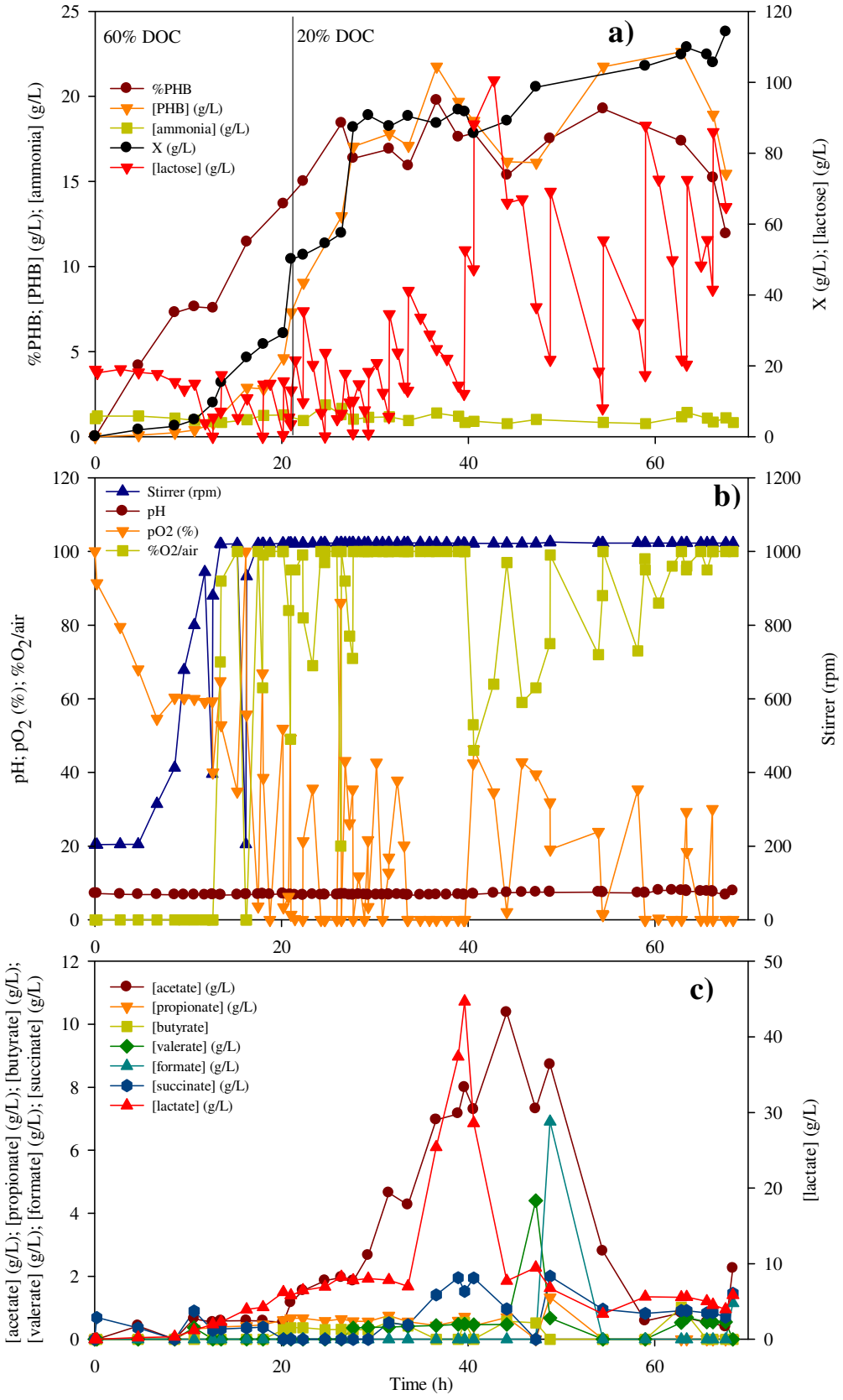

Figure 16 - PHB production from whey in MR medium by *E. coli* CML 3-1, in fed-batch system with manual feeding and oxygen limitation; a) PHB content and PHB, active biomass, ammonia and lactose concentrations, b) pH, pO_2, $\%O_2$/air and stirrer, c) organic acids concentration

The feeding strategy imposed in this experiment resulted well in the previous assays (see section 4.3.3) but presented some drawbacks when the operational time was increased. As expected, a very high biomass concentration was obtained but it resulted in a high lactose consumption rate. Between 20 h of operation time and the end of exponential phase, it was very difficult to keep lactose above 5 g/L. In order to face this increase on lactose consumption rate, a further experiment, with a continuous feeding strategy, was planned. Moreover, the increase on active biomass concentration also contributed to higher oxygen consumption. Consequently it was difficult to keep DOC control near 60%. This control was especially difficult during exponential phase growth and DOC reached 0% several times. This oxygen limitation resulted in lactose fermentation to organic acids during the growth and storage phases. As could be observed on Figure 16c, during oxygen depletion, organic acids were produced mainly lactate and acetate (Wong et al., 1999). The organic acids formation resulted from the metabolic pathways that microorganisms utilize when oxygen become limiting. Without an external electron acceptor, lactose is converted to organic acids instead of cell growth or PHB production (Wong et al., 1999; Phue et al., 2005). After some time microorganisms started to consume the organic acids produced (Figure 16c). The mechanisms that control organic acids formation and consumption should be clarified since their presence in the medium can influence cell growth and PHB production.

Contrary to the observed in fed-batch experiment with pH feed control (see section 3.3.2), DOC limitation at OD_{600nm} of 100.5 (with a dilution factor of 1:500) did not seamed to affect both polymer storage and biomass production. As could be seen on Figure 16a and c, after DOC limitation to 20%, PHB storage and active biomass concentration continued to increase and higher amounts of organic acids production were detected.

4.3.5 – Fed-batch experiment with continuous feeding

In this experiment, a fed-batch system with continuous feeding was implemented in order to overcome the problems found in the previous assay (Section 4.3.4). The feeding rates used in this experiment were determined in order to have the same lactose consumption rates obtained in the last experiment and, in order to avoid the lactose depletion in case of high consumption rate. Based on average consumption rates of each phase growth, the strategy implemented was the following: in the beginning of the

experiment a pulse of 30 g/L was added manually, then when lactose concentration reached 24 g/L, the continuous feeding was switched on at a flow rate of 15 g/L/h in exponential phase. (Table 9, in Appendix 8.3.4). The evolution of organic acids, lactose, ammonia, PHB and biomass concentration, as well as PHB content, pH, DOC (pO_2), $\%O_2$/air and stirring observed in this experiment are shown in Figure 17.

In the final of this experiment 63.02% of PHB content, 16.21 g/L of maximum PHB concentration (both achieved at 131 h) and 23.99 g/L of maximum active biomass concentration (achieved at 17.63 h) were achieved. The storage and growth yields were 0.13 $g_{PHB}/g_{lactose}$ and 0.075 $g_X/g_{lactose}$, respectively. The volumetric productivity was 0.12 g_{PHB}/L.h, the specific productivity was 0.015 g_{PHB}/g_X.h and the maximum specific growth rate was 0.24 h^{-1} (Figure 30, in appendix 8.3.5).

The results obtained (Figure 17a and Table 8) showed that with the continuous feeding strategy, the maximum active biomass concentration (23.99 g/L) was the lowest of all the fed-batch experiments performed with cheese whey. The maximum specific growth rate (0.24 h^{-1}) was lower than the experiment performed with manual feeding strategy (0.40 h^{-1}). The lower growth rate led to lower biomass concentration, due to the high lactose concentration accumulated in reactor, since the beginning of the continuous feed. Giving that OD_{600nm} did not reach 100, oxygen limitation was not applied as it was programmed. Despite this higher PHB storage, the volumetric productivity (0.12 g_{PHB}/L.h) obtained still far from the 2.57 g_{PHB}/L.h obtained by Ahn and co-workers (2000) (Table 8). Since this fed-batch experiment did not have a limitation in oxygen concentration, the absence of an oxygen limitation appliance could be one of the reasons for this productivity difference.

Regarding PHB production, even without applying oxygen limitation, the strategy revealed to be successful since in this experiment the highest PHB content was obtained (63.0%). Until 33 h of operation time, a high PHB content (50%) was obtained. This increase in PHB production was more evident especially after lactose accumulated in the medium reached near 150 g/L. This indicates that lactose consumed was most used for PHB production instead of growth.

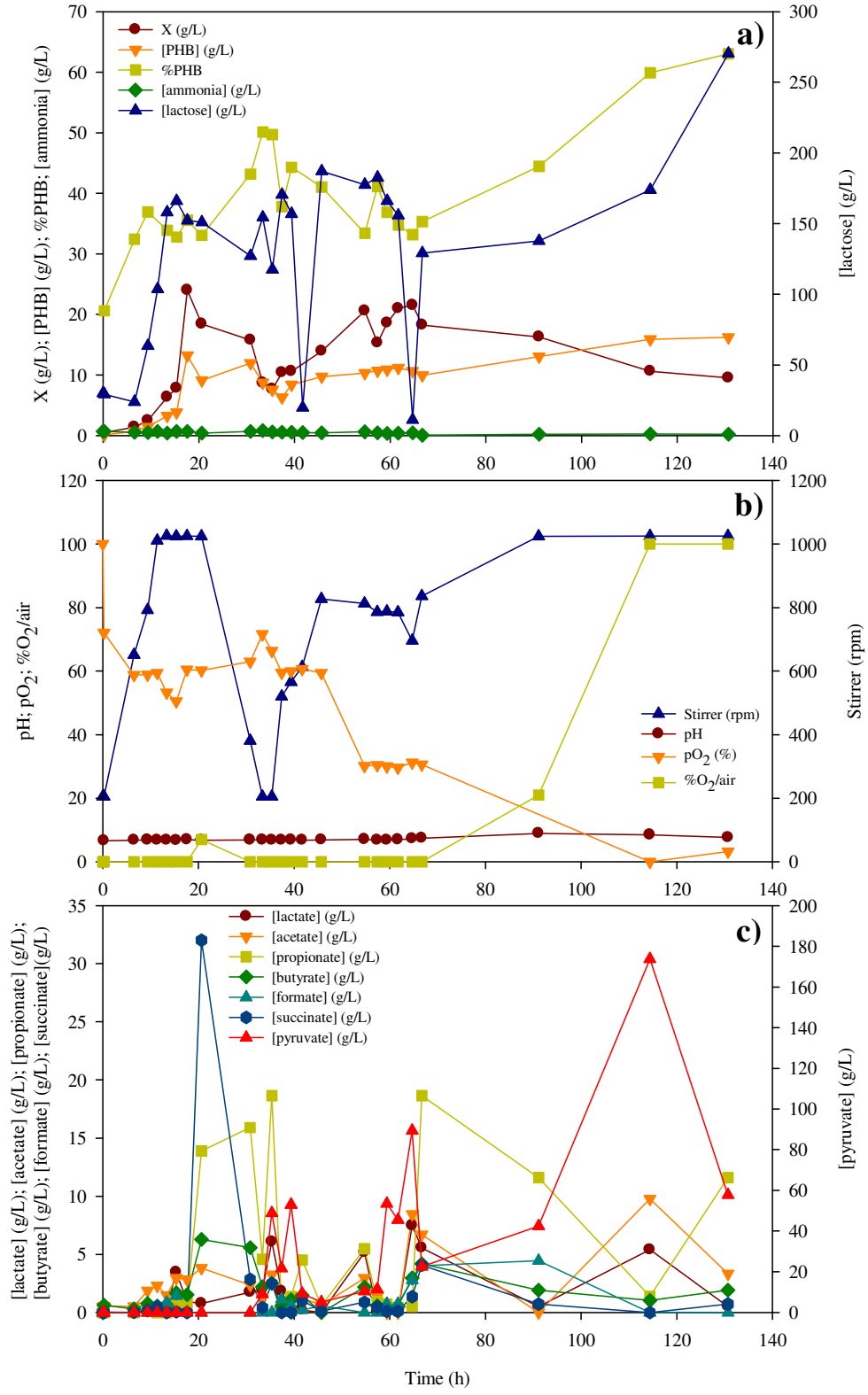

Figure 17 - PHB production from whey powder in mineral medium by *E. coli* CML 3-1, in fed-batch system with continuous feeding a) PHB content and PHB, active biomass, ammonia and lactose concentrations, b) pH, pO_2, $\%O_2$/air and stirrer, c) organic acids concentration

Between 17 h and 40 h of operation time, active biomass concentration decreased and a significant increase in PHB content was observed. The excess of lactose (more than 150 g/L) or the metabolic products (such as pyruvate) accumulated inside the cells during this time, could lead to cells lysis (Figure 17a and c). The pyruvate produced probably results from the excess of lactose in the external medium (Figure 17a and c). In *E. coli*, carbon source excess can lead to an intracellular pyruvate accumulation. Pyruvate could be released to the external medium or could be converted into lactate. (Wong et al., 1999). Moreover this situation inhibits cell growth and favours PHB production (Wong et al., 1999).

Between 39 h-46 h and 59 h-65 h of operation time, lactose concentration decreased, because in these periods feeding rate was stopped in order to avoid a higher lactose accumulation. When lactose addition was stopped this coincided with pyruvate release to the external medium. After releasing pyruvate, the inhibition of cell growth disappeared and the formation of active biomass was observed (Figure 17a).

After 65 h and until the end of this experiment, it was observed, a new effective increase of PHB content with simultaneous production and posterior consumption of large concentrations of pyruvate. This was observed when lactose was again accumulated in excess in medium. This could be one of the causes for cell disruption observed at the end of this experiment (Figure 18). Since cellular membrane of *E. coli* is very fragile, this could be disrupted due the high content of polymer or due to the high concentrations of lactose and other nutrients in the external medium or metabolites produced intracellularly. Cell membrane disruption, in the final of the process, is extremely advantageous for the downstream processing. Since the extraction of the polymer from the cell is one of the most expensive steps of the process.

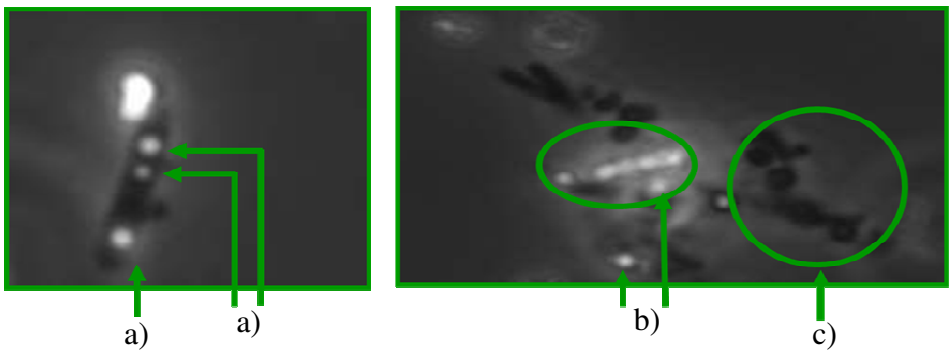

Figure 18 - Stained sample of *E. coli* CML 3-1 strain in fed-batch experiment with continuous feeding: a) polymer granules inside the cell; b) polymer granules outside the cell; c) cellular debris (1000x)

5. Conclusions

The cost effectiveness of PHAs production is the main factor that prevents the use of these biopolymers as biodegradable commonly plastics. The use of renewable feedstocks and the establishment of novel and better PHA synthesis strategies involving high producing strains such as genetic engineering *E. coli* can largely contribute to reducing production global costs.

In this work, a process for PHB production from cheese whey, a cheap by-product of cheese industry, in a fed-batch system using recombinant *E. coli* harbouring *C. necator* PHB synthesis genes was developed. The main conclusions from this work are the following:

- Thought all tested *E. coli* strains being able to produce PHB, CML 3-1 was the strain with the best polymer producing performance.
- Diverse feeding strategies were evaluated, in order to improve both cell growth and PHB production (since lactose induces both growth and polymer storage). From all strategies tested, the feeding controlled by pH seemed to be the best strategy, in spite of the operational problems with lactose and oxygen concentration depletion and the possible organic acids production. In this experiment the best storage and production yields (0.14 $g_{PHB}/g_{lactose}$ and 0.50 $g_X/g_{lactose}$, respectively), the highest volumetric productivity (0.57 g_{PHB}/L.h) and PHB concentration (33.76 g/L), were obtained.
- Despite of the promising results obtained, the system performance should be optimized since the PHB storage content is still lower than one of the best results published in the literature (80% of PHB content obtained by Ahn and co-workers (2000)). The optimization should focus the following aspects: a feeding strategy that avoids lactose depletion, introduction of oxygen limitation only when lactose concentration was in high amounts for PHB production enhancing and the understanding of ammonia's role on PHB production.
- The last experiment with continuous feeding strategy confirmed that recombinant *E. coli* can disrupt easily contributing for a simpler and cheaper PHB extraction process and reducing the costs of downstream processing. However, the cell disruption occurred in an early stage of the process, which probably resulted from the accumulation of lactose or metabolites in the culture medium.

5.1 - Future Strategies

The objective of this work was the optimization of PHB production by recombinant *E. coli* from cheese whey. However the results regarding the production of PHB obtained were still lower than those obtained in the literature (Table 8). In this way, the following strategies should be implemented.

5.1.1 – Carbon source feeding strategy

From the results obtained it seemed that growth and PHB production should be separated with the first stage for biomass growth (with a little polymer production, since lactose induces both growth and PHB storage) and, the second one for polymer production (inhibiting cells growth). This feeding strategy should avoid lactose depletion in culture.

The reactor working in a fed-batch mode should be operated differently in the two stages. In the first stage, a feeding strategy by pulses with low lactose concentration, similar to which was used in section 4.3.4, should be applied. In this way it could be possible to achieve high cell concentration during growth phase. The transition to polymer production phase should occur when a high cell concentration would be reached. A continuous feeding similar to the continuous feeding strategy used in this work, in section 4.3.5 should be applied. By keeping high lactose concentrations, PHB-synthesis is promoted and high PHB storage should be achieved.

5.1.2 – Oxygen limitation and ammonia role

In this study, excess lactose concentration was the main factor to achieve high polymer storage content. If DOC limitation to 10% or 20% was applied immediately after the continuous feeding implementation, during PHB storage phase, the enhancing of polymer production is expected to be even higher. This limitation must be imposed after continuous feeding, in order to guarantee a high lactose concentration. As was already seen in fed-batch experiment with feed control by pH (see section 4.3.2), if oxygen limitation takes place during biomass growth phase, PHB production and biomass growth will be negatively affected (Figure 13) (Wong et al., 1999). However the role of oxygen and its absence in cell metabolism should be further understood in order to determine the precise moment and DOC concentration that should be imposed.

Attention should be also devoted to the possibility of lactose fermentation to organic acids in situations with limiting DOC concentration.

Besides oxygen, the role of ammonia in PHB storage by *E. coli* should also be further elucidated. As could be observed in the fed-batch experiment where was achieved the highest cell concentration (see section 4.3.4), the addiction of ammonia due to pH control stimulates cells growth. So, during the growth phase, a supply of ammonia in feed solution is probably needed, since low concentrations of ammonia could lead to the lost of cells viability. During PHB storage phase, ammonia concentration should be lower than in growth phase, since high ammonia concentrations stimulate cell growth instead of polymer storage. But, this concentration should not be too much lower due to cells viability maintenance. Taking into account the cheese whey flask experiments results (Figure 12 in section 4.3.1), for ammonia concentrations between 0.6 g/L and 0.03 g/L, cell growth was inhibited while PHB content increased. This means that it is necessary to find a value of X/N ratio that enhance polymer storage and maintain cells viability at the same time.

The ammonia strategy presented should be taken in a fed-batch system but with a different base solution (such as NaOH) for pH control. In order to completely understand the real role of ammonia on PHB storage, recombinant *E. coli* CML 3-1 should to be tested with different ammonia concentrations, during storage phase.

6. References

Ahn, W. S., Park, S. J., Lee, S. Y. (2000). Production of Poly(3-Hydroxybutyrate) by Fed-Batch Culture of Recombinant *Escherichia coli* with a Highly Concentrated Whey Solution. Applied and Environmental Microbiology, 66 (8), 3624–3627.

Ahn, W. S., Park, S. J., Lee, S. Y. (2001). Production of poly(3-hydroxybutyrate) from whey by cell recycle fed-batch culture of recombinant *Escherichia coli*, Biotechnology Letters, 23, 235–240.

Biby, G.D. Degradable polymers. International Card Manufacture Association. http://www.icma.com/info/polymers.htm (consulted at 25/10/09).

Blaschek, K. M., Wendorff, W. L., Rankin, S. A. (2007). Survey of Salty and Sweet Whey Composition from Various Cheese Plants in Wisconsin. Journal of Dairy Science, 90 (4), 2029–2034.

Braunegg, G., Sonnleither, B., Lafferty, R. M. (1978); A rapid gas chromatographic method for the determination of poly-β- hydroxybutyric acid in microbial biomass. European Journal of Applied Microbiology and Biotechnology, 6, 29-37.

Choi, J., Lee, S. Y. (1997). Process analysis and economic evaluation for Poly(3-hydroxybutyrate) production by fermentation. Bioprocess Engineering, 17, 335–342.

Comeau, Y., Hall, K.J., Oldham, W.K. (1998) Determination of poly-β-hydroxybutyric in activated sludge by gas-liquid chromatography. Applied Environmental Microbiology, 59, 2325-2327.

Corchero, J. L., Cubarsí, R., Vila, P., Arís, A., Villaverde, A. (2001). Cell lysis in *Escherichia coli* cultures stimulates growth and biosynthesis of recombinant proteins in surviving cells. Microbiological Research 156**,** 13–18.

Harding, K.G., Dennis, J.S., von Blottnitz, H., Harrison, S.T.L. (2007). Environmental analysis of plastic production processes: Comparing petroleum-based polypropylene and polyethylene with biologically-based poly-β-hydroxybutyric acid using life cycle analysis. Journal of Biotechnology, 130, 57–66.

Holmes, P.A., Lim, G.B. (1984). Separation process. European Patent: 145233.

Instituto Nacional de Estatística (2008). Estatísticas agrícolas 2008.

Jau, M.-H., Yewa, S.P., Toh, P. S. Y., Chonga, A. S. C., Chub, W.-L., Phang, S.-M., Najimudin, N., Sudesh, K. (2005). Biosynthesis and mobilization of poly(3-

hydroxybutyrate) [P(3HB)] by *Spirulina platensis*. International Journal of Biological Macromolecules, 36, 144–151.

Kahara, P., Tsugea, T., Taguchib, K., Doia, Y. (2004). High yield production of polyhydroxyalkanoates from soybean oil by *Ralstonia eutropha* and its recombinant strain. Polymer Degradation and Stability, 83, 79–86.

Kim, B. S. (2000). Production of poly(3-hydroxybutyrate) from inexpensive substrates. Enzyme and Microbial Technology, 27, 774–777.

Kim, B. S., Lee, S. Y., Chang, H. N. (1992). Production of poly-β-hydroxybutyrate by fed-batch culture of recombinant *Escherichia coli*. Biotechnology Letters, 14(9), 811-816.

Koller, M., Bona, R., Chiellini, E., Fernandes, E. G., Horvat, P., Kutschera, C., Hesse, P., Braunegg, G. (2008). Polyhydroxyalkanoate production from whey by *Pseudomonas hydrogenovora*. Bioresource Technology, 99, 4854– 4863.

Koller, M., Hesse, P., Bona, R., Kutschera, C., Atlić, A., Braunegg, G. (2007). Potential of various Archae- and Eubacterial strains as industrial polyhydroxyalkanoate producers from whey. Macromolecular Bioscience, 7, 218– 226.

Lee, S. Y. (1996). Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria. TIBTECH, 14, 431-438.

Lee, S.Y., Middelberg, A. P. J., Lee Y. K. (1997). Poly(3-hydroxybutyrate) production from whey using recombinant *Escherichia coli*. Biotechnology Letters, 19 (10), 1033–1035.

Lemoigne, M. (1926). Produits de dehydration et de polymerisation de l'acide ß-oxobutyrique. Bull Soc Chim Biol, 8, 770–782.

Li, R., Zhang, H., Qi, Q. (2007). The production of polyhydroxyalkanoates in recombinant *Escherichia coli*. Bioresource Technology, 98, 2313–2320.

Ling, Y., Wong, H.H., Thomas, C.J., Williams, D.R.G., Middelberg, A.P.J. (1997). Pilot-scale extraction of PHB from recombinant *E. coli* by homogenization and centrifugation. Bioseparation, 7, 9–15.

Luengo, J. M., García, B., Sandoval, A., Naharroy, G., Olivera, E. R. (2003). Bioplastics from microorganisms. Current Opinion in Microbiology, 6, 251–260.

Madison, L., Huisman, G. (1999). Metabolic engineering of poly(3-Hydroxyalkanoates): from DNA to plastic. Microbiology and Molecular Biology Reviews, 63 (1), 21–53.

Morrow, T. (2007). Revolutionary heart valves, sutures from miracle substance-polyester. Managed Care Magazine, http://www.managedcaremag.com/archives/0703/0703.biotech.html (consulted at 25/10/09).

Park, S. J., Park, J. P., Lee, S. Y. (2002). Production of poly(3-hydroxybutyrate) from whey by fed-batch culture of recombinant *Escherichia coli* in a pilot-scale fermenter. Biotechnology Letters, 24, 185–189.

Phue, J.-N., Noronha, S. B., Hattacharyya, R., Wolfe, A. J., Shiloach, J. (2005). Glucose metabolism at high density growth of *E. coli* B and *E. coli* K: differences in metabolic pathways are responsible for efficient glucose utilization in *E. coli* B as determined by microarrays and northern blot analyses. Biotechnology and Bioengineering, 90 (7), 805-820.

Pohlmann, A., Fricke, W. F., Reinecke, F., Kusian, B., Liesegang, H., Cramm, R., Eitinger, T., Ewering, C., Pötter, M., Schwartz, E., Strittmatter, A., Vo, I., Gottschalk, G., Steinbüchel, A., Friedrich, B., Bowien, B. (2006). Genome sequence of the bioplastic-producing "Knallgas" bacterium *Ralstonia eutropha* H16. Nature Biotechnology, 24, 1257–1262.

Ramsay, J. A., Berger, E., Ramsay, B. A., Chavarie, C. (1990). Recovery of poly-3-hydroxyalkanoic acid granules by a surfactant-hypochlorite treatment. Biotechnology Techniques, 4(4), 221–226.

Reddy, C. S. K., Ghai, R., Rashmi, Kalia, V. C. (2003). Polyhydroxyalkanoates: an overview. Bioresource Technology, 87, 137–146.

Reis, M.A.M., Serafim, L.S., Lemos, P.C., Ramos, A.M., Aguiar, F.R., van Loosdrecht, M.C.M. (2003). Production of polyhydroxyalkanoates by mixed microbial cultures. Bioprocess Biosystems Engineering, 25, 377–385.

Satoh, H., Mino, T., Matsuo, T. (1992). Uptake of organic substrates and accumulation of polyhydroxyalkanoates linked with glycolysis of intracellular carbohydrates under anaerobic conditions in the biological excess phosphate removal processes. Water Science and Technology, 26, 933-942.

Siso, M. I. González (1996). The biotechnological utilization of cheese whey: a review. Bioresource Technology, 57, 1-11.

Solaiman, D. K. Y., Ashby, R. D., Foglia, T. A., Marmer, W. N. (2006). Conversion of agricultural feedstock and coproducts into poly(hydroxyalkanoates). Applied Microbiology and Biotechnology, 71, 783–789.

van Wegen, R. J., Lee, S. Y., Middelberg, A. P. J. (2001). Metabolic and Kinetic Analysis of Poly(3-Hydroxybutyrate) Production by Recombinant *Escherichia coli*. Biotechnology and Bioengineering, 74(1), 70-80.

Vandamme, P., Coenye, T. (2004). Taxonomy of the genus Cupriavidus: a tale of lost and found. International Journal of Systematic and Evolutionary Microbiology, 54, 2285–2289.

Verlinden, R. A. J., Hill, D. J., Kenward, M. A., Williams, C. D., Radecka, I. (2007). Bacterial synthesis of biodegradable polyhydroxyalkanoates. Journal of Applied Microbiology, 102, 1437-1449.

Wendlandt, K.-D., Geyer, W., Mirschel, G., F. Hemidi, F. A.-H. (2005). Possibilities for controlling a PHB accumulation process using various analytical methods. Journal of Biotechnology, 117, 119–129.

Williams, S. F., Martin, D.P., Horowitz, D.M., Peoples, O.P., (1999). PHA applications: addressing the price performance issue. I. Tissue engineering. International Journal of Biological Macromolecules, 25, 111–121.

Wong, H. H., van Wegen, R. J., Choi, J.-I., Lee, S. Y. (1999). Metabolic analysis of poly(3-hydroxybutyrate) production by recombinant *Escherichia coli*. Journal of Microbiology and Biotechnology, 9(5), 593-603.

Wong, P. A. L., Cheung, M. K., Lo, W.-H., Chua, H., Yu, P. H. F. (2004). Investigation of the effects of the types of food waste utilized as carbon source on the molecular weight distributions and thermal properties of polyhydroxy-butyrate produced by two strains of microorganisms. e-Polymers, 31. http://www.e-polymers.org (consulted at 31/08/09).

Yamanaka, K., Kimura, Y., Aoki, T., Kudo, T. (2009). End-group analysis of bacterially produced poly(3-hydroxybutyrate): discovery of succinate as the polymerization starter. Macromolecules, 42, 4038-4046.

Zinn, M., Witholt, B., Egli, T. (2001). Occurrence, synthesis and medical application of bacterial polyhydroxyalkanoate. Advanced Drug Delivery Reviews, 53, 5–21.

Zydney, A. L. (1998). Protein separation using membrane filtration: new opportunities for whey fractionation. International Dairy Journal, 8, 243–250.

7. Publications

L.S. Serafim, D. Contu, I. Farinha, J. Pais, P.C. Lemos, M.A.M. Reis; "BIOPLASTICS PRODUCTION FROM CHEESE WHEY BY H. MEDITERRANEI". RRB5 – International Conference on Renewable Resources and Biorefineries, Gent, Belgium, 10-12 June, 2009. (Best Poster Award)

J. Pais, L.S. Serafim, I. Farinha, M.A. Prieto, M. Arévalo-Rodríguez, M.A.M. Reis; "BIOPLASTICS PRODUCTION FROM CHEESE WHEY BY RECOMBINANT E. COLI". RRB5 – International Conference on Renewable Bioresources and Biorefineries, Gent, Belgium, 10-12 June, 2009.

J. Pais, L.S. Serafim, I. Farinha, M.A. Prieto, M. Arévalo-Rodríguez, M.A.M. Reis; "BIOPLASTICS PRODUCTION FROM CHEESE WHEY BY RECOMBINANT E. COLI". ECB14 - 14th European Congress on Biotechnology, Barcelona, Spain, 13-16 September, 2009.

J. Pais, L.S. Serafim, I. Farinha, M.A. Prieto, M. Arévalo-Rodríguez, M.A.M. Reis; "Optimization of polyhydroxyalkanoates production from cheese whey by recombinant E. coli". ESBP 2009 - 5th European Symposium on Biopolymers, Funchal, Portugal, 18-19 November, 2009.

J. Pais, L.S. Serafim, I. Farinha, M.A. Prieto, M. Arévalo-Rodríguez, M.A.M. Reis; "Polyhydroxybutyrate production from cheese whey by recombinant E. coli". III International Conference on Environmental, Industrial and Applied Microbiology (BioMicroWorld2009), Lisbon, Portugal, 2-4 December, 2009.

8. Appendix

8.1 – LB medium experiments

8.1.1 - Selection of the Strain

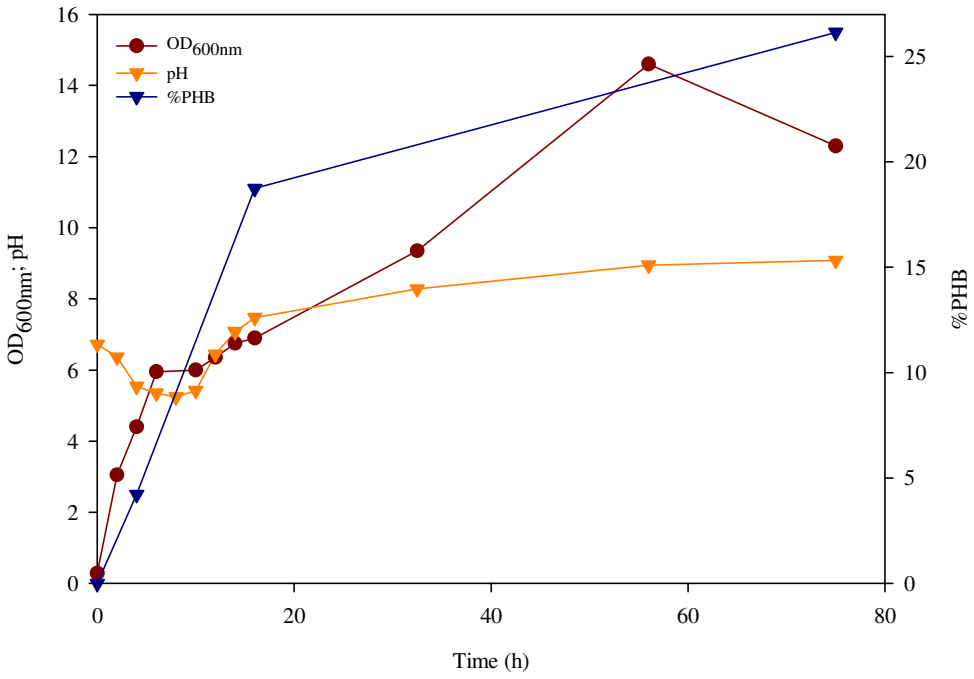

Figure 19 – PHB production from lactose in LB medium with *E. coli* CML 1-1A

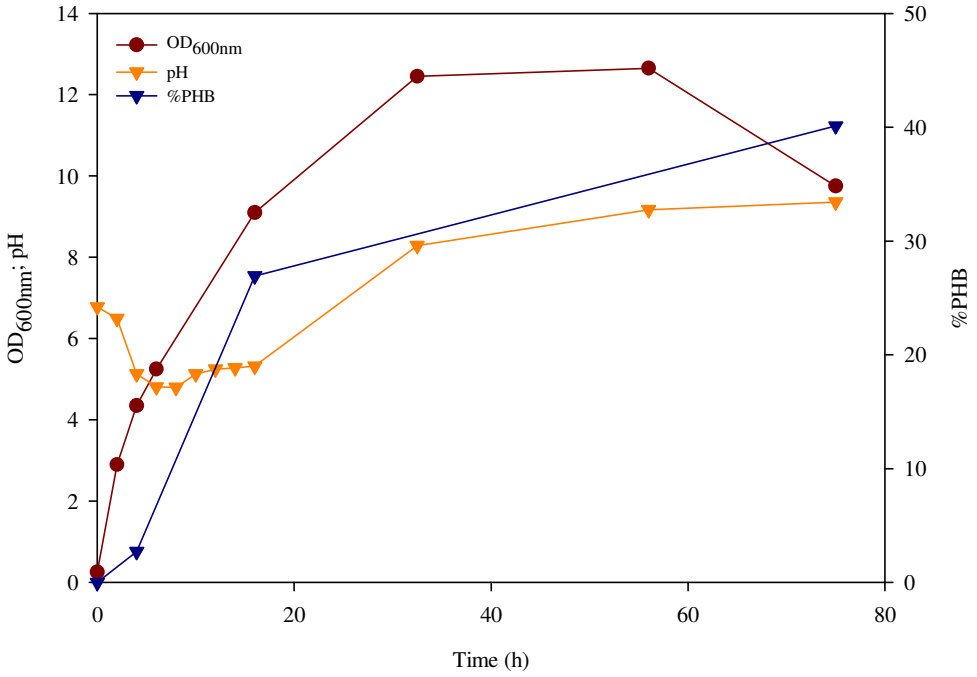

Figure 20 - PHB production from lactose in LB medium with *E. coli* CML 2-3A

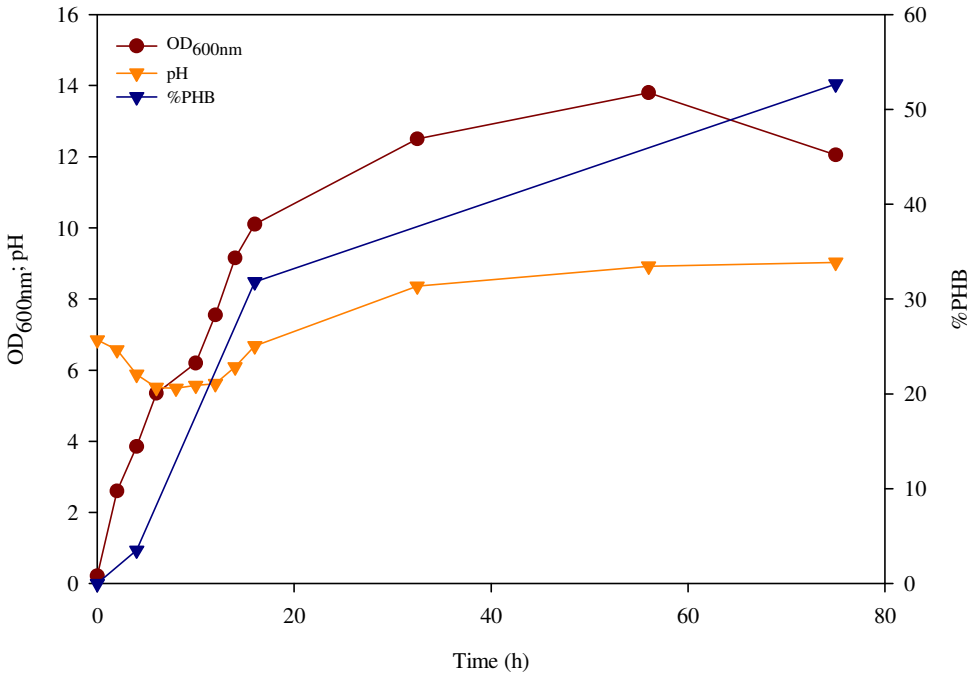

Figure 21 - PHB production from lactose in LB medium with *E. coli* CML 3-1

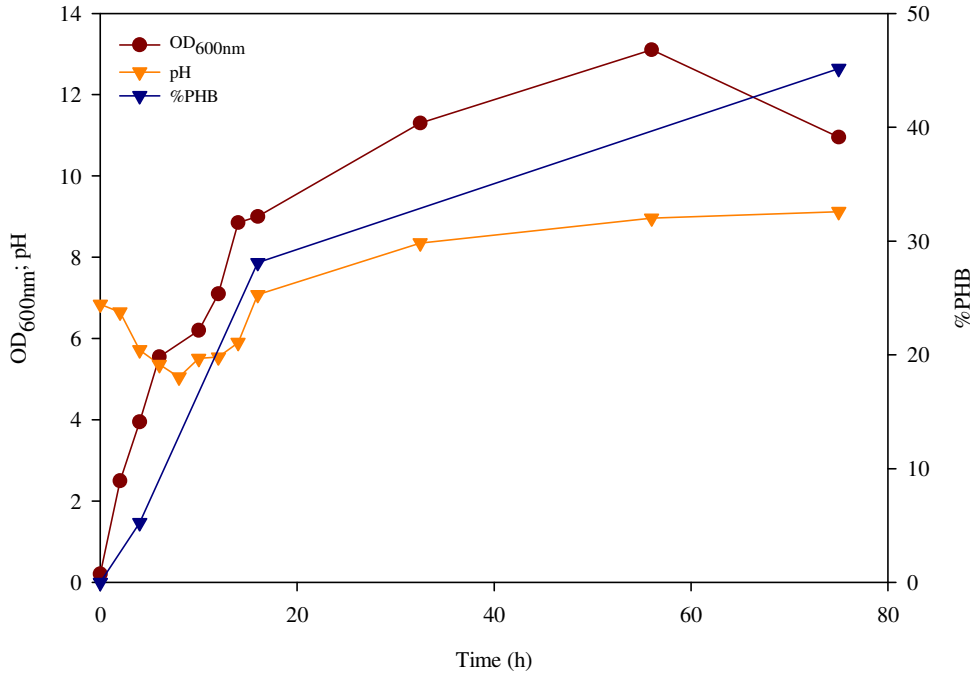

Figure 22 - PHB production from lactose in LB medium with *E. coli* CML 3-2A

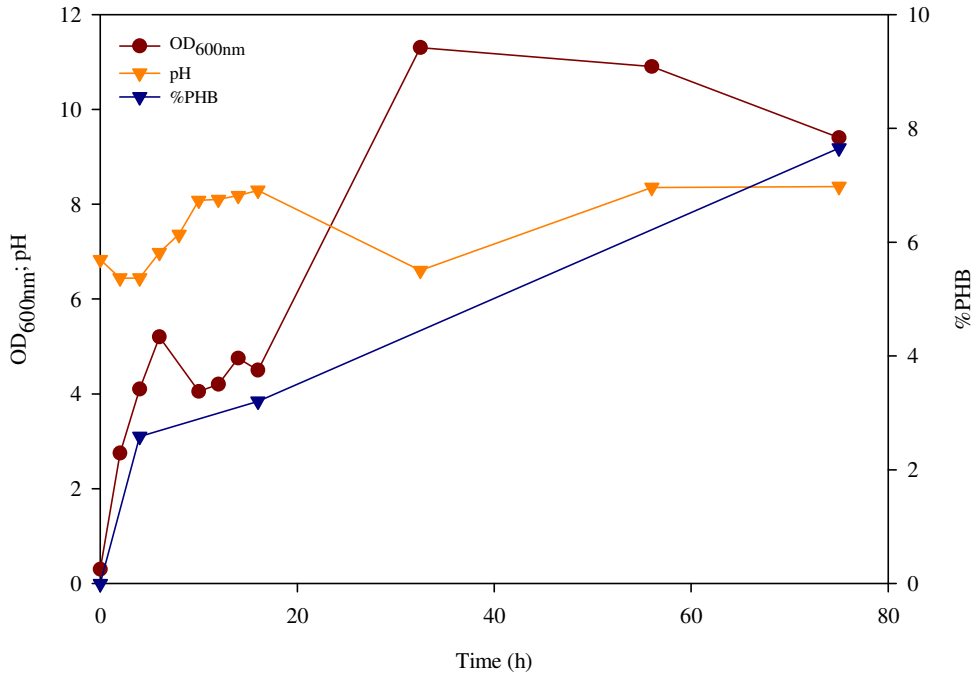

Figure 23 - PHB production from lactose in LB medium with *E. coli* CML 4-1A

8.1.2 - Flask experiment with *E. coli* CML 3-1 strain

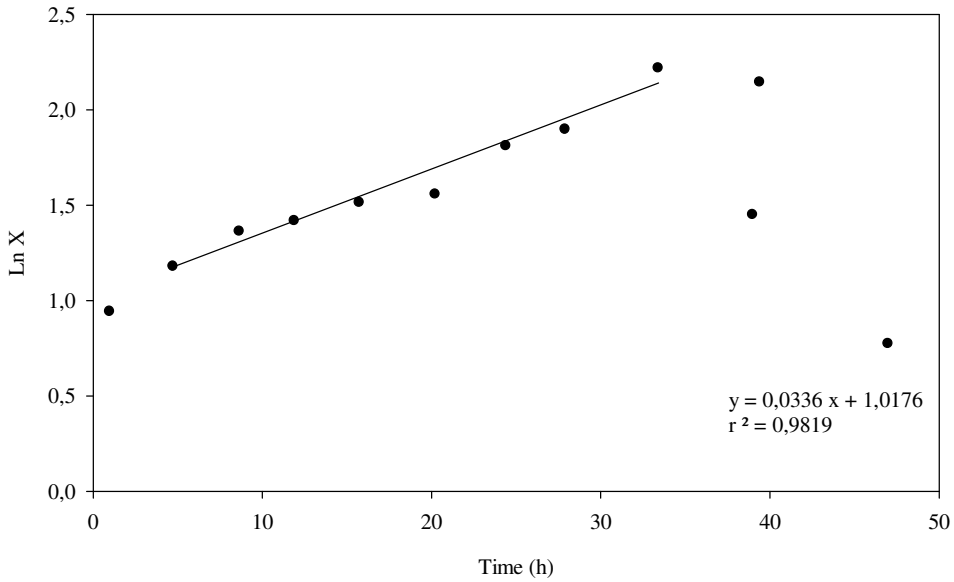

Figure 24 - Specific growth rate determination for *E. coli* CML 3-1, in shake flask with LB medium with lactose

8.2 – Defined medium with lactose experiments – fed-batch system

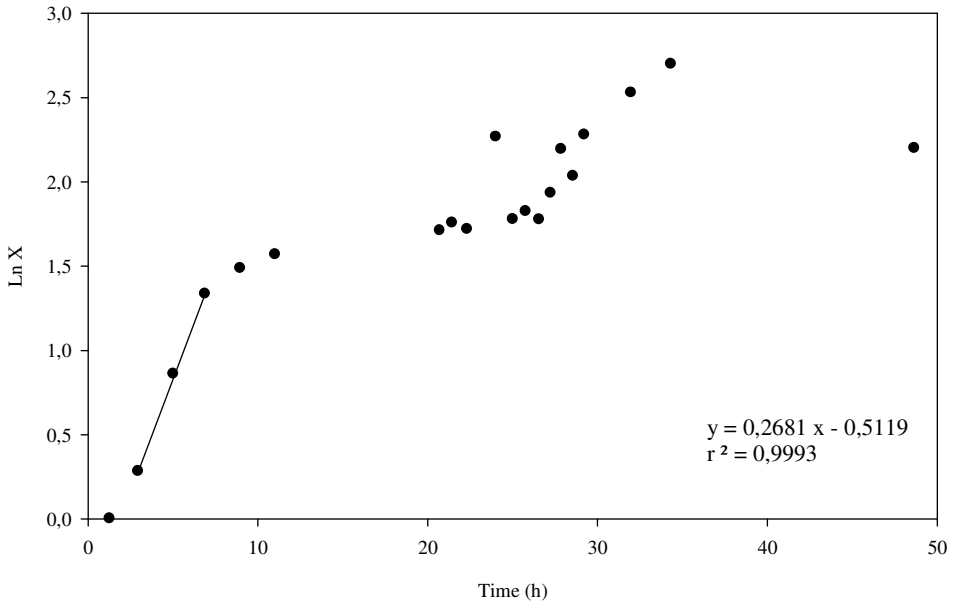

Figure 25 – Specific growth rate determination for *E. coli* CML 3-1, in fed-batch experiment with lactose and MR medium

8.3 – Defined medium experiments with cheese whey

8.3.1 – Flask experiment

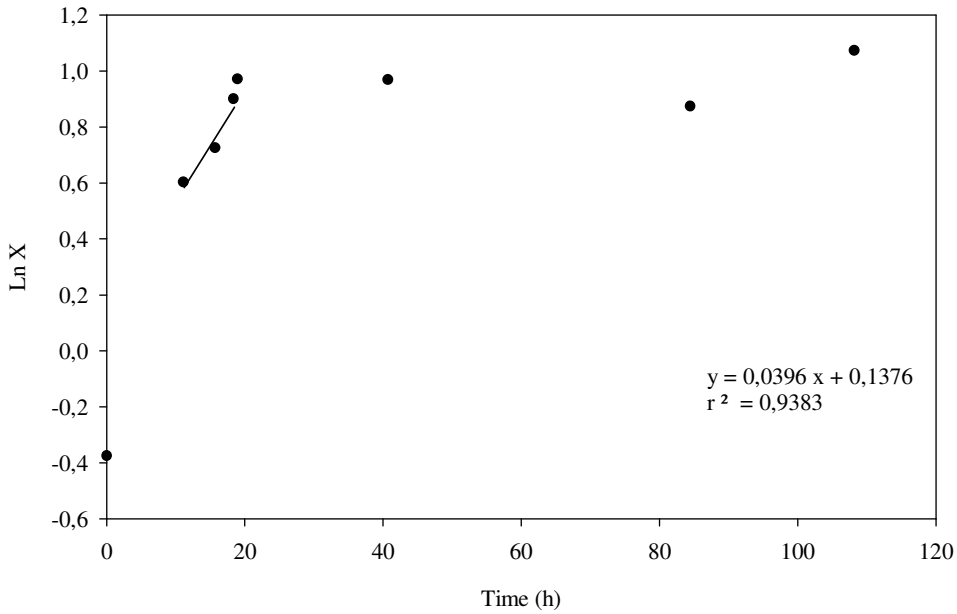

Figure 26 - Specific growth rate determination for *E. coli* CML 3-1, in shake flask

8.3.2 – Fed-batch experiment with feeding controlled by pH

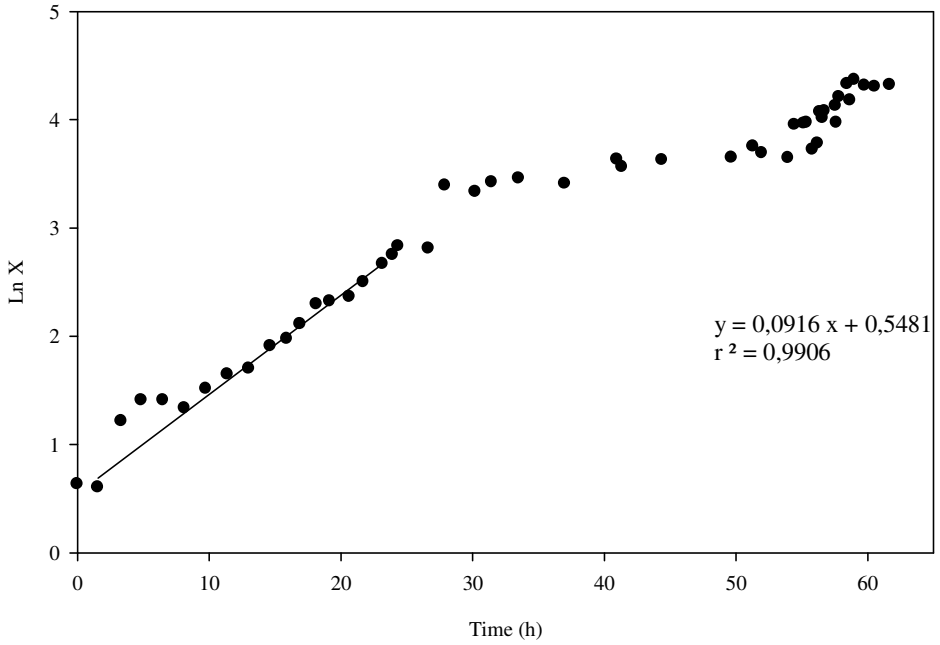

Figure 27 - Specific growth rate determination for *E. coli* CML 3-1, in fed-batch experiment with feeding controlled by pH

8.3.3 – Fed-batch experiment with manual feeding

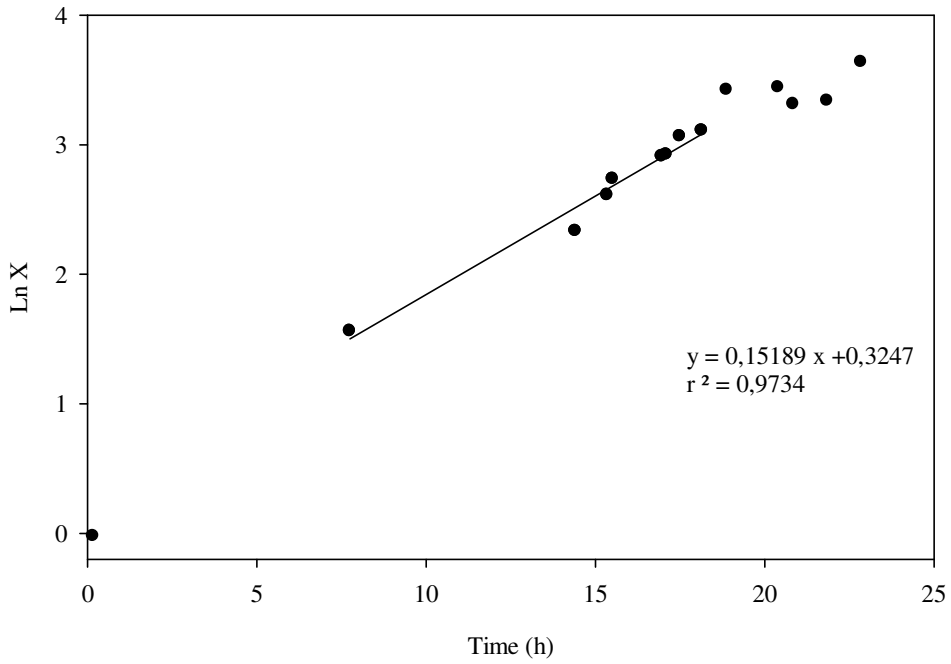

Figure 28 - Specific growth rate determination for *E. coli* CML 3-1, in fed-batch experiment with manual feeding

8.3.4 - Fed-batch experiment with manual feeding and oxygen limitation

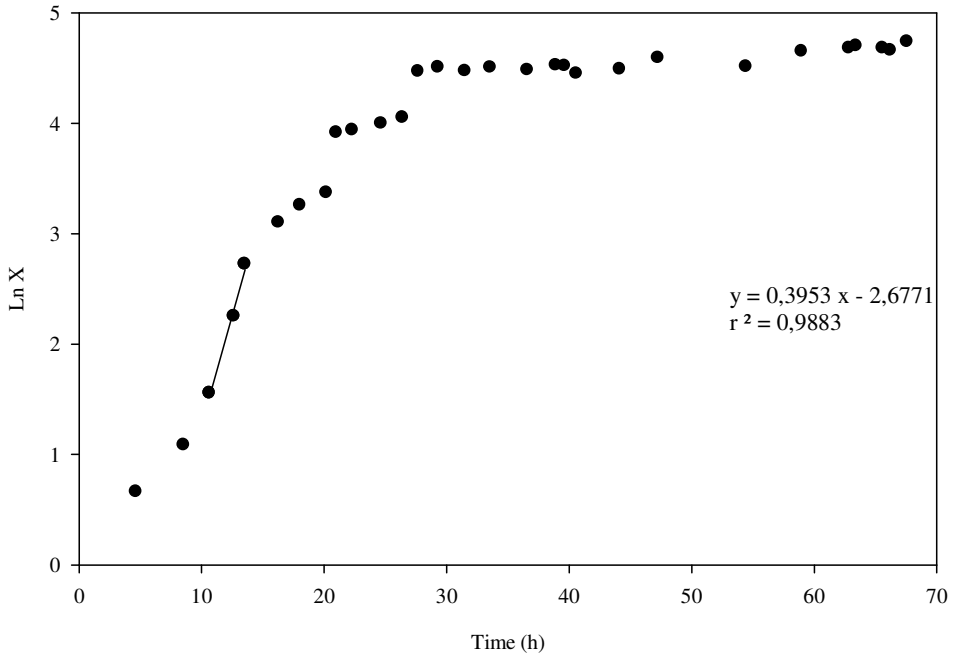

Figure 29 - Specific growth rate determination for *E. coli* CML 3-1, in fed-batch experiment with manual feeding and oxygen limitation

Table 9 – Lactose consumption rate determination for fed-batch experiment with manual feeding and oxygen limitation

initial time (h)	[lactose]initial (g/L)	final time (h)	[lactose]final (g/L)	consumption rate (g/L/h)	consumption rate average (g/L/h)
Lag phase					1
0,00	18,87	11,78	3,79	1	
Exponential phase					15
13,53	29,06	15,27	5,24	14	
18,05	14,75	20,13	0,45	7	
20,20	18,78	20,97	3,51	20	
21,02	26,48	22,27	9,70	13	
22,32	35,40	24,62	0,07	15	
24,67	23,62	25,93	4,96	15	
26,80	17,75	27,62	0,90	21	
28,27	14,81	29,27	0,84	14	
Stationary phase					11
29,30	18,29	31,47	5,60	6	
31,52	34,55	33,57	12,97	11	
34,87	33,53	39,53	12,08	5	
39,63	52,59	40,55	47,22	6	
40,60	88,11	48,77	21,70	8	
48,80	69,06	54,38	8,00	11	
54,45	55,36	58,93	17,36	8	
58,98	87,75	63,38	20,32	15	
63,43	72,46	66,23	41,45	11	
67,60	85,94	68,38	64,76	27	

8.3.5 - Fed-batch experiment with continuous feeding

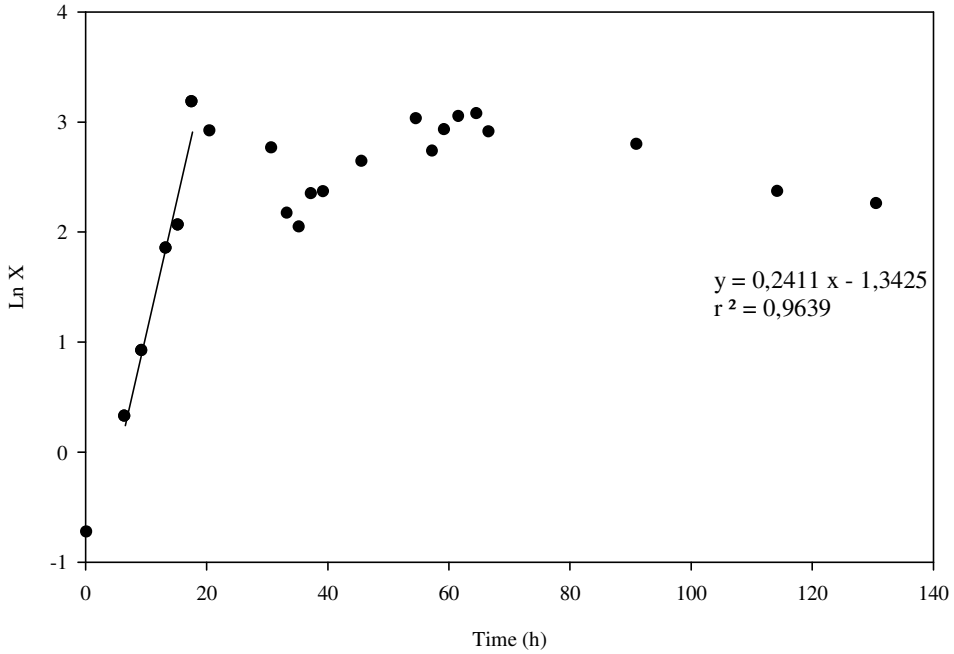

Figure 30 - Specific growth rate determination for *E. coli* CML 3-1, in fed-batch experiment with continuous feeding